

## REVIEW ARTICLE

## Genetic polymorphism in metabolism and host defense enzymes: Implications for human health risk assessment

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**Abstract**

Genetic polymorphisms in xenobiotic metabolizing enzymes can have profound influence on enzyme function, with implications for chemical clearance and internal dose. The effects of polymorphisms have been evaluated for certain therapeutic drugs but there has been relatively little investigation with environmental toxicants. Polymorphisms can also affect the function of host defense mechanisms and thus modify the pharmacodynamic response. This review and analysis explores the feasibility of using polymorphism data in human health risk assessment for four enzymes, two involved in conjugation (uridine diphosphoglucuronosyltransferases [UGTs], sulfotransferases [SULTs]), and two involved in detoxification (microsomal epoxide hydrolase [EPHX1], NADPH quinone oxidoreductase I [NQO1]). This set of evaluations complements our previous analyses with oxidative and conjugating enzymes. Of the numerous UGT and SULT enzymes, the greatest likelihood for polymorphism effect on conjugation function are for *SULT1A1* (\*2 polymorphism), *UGT1A1* (\*6, \*7, \*28 polymorphisms), *UGT1A7* (\*3 polymorphism), *UGT2B15* (\*2 polymorphism), and *UGT2B17* (null polymorphism). The null polymorphism in *NQO1* has the potential to impair host defense. These highlighted polymorphisms are of sufficient frequency to be prioritized for consideration in chemical risk assessments. In contrast, SNPs in *EPHX1* are not sufficiently influential or defined for inclusion in risk models. The current analysis is an important first step in bringing the highlighted polymorphisms into a physiologically based pharmacokinetic (PBPK) modeling framework.

**Keywords:** Epoxide hydrolase; glucuronosyltransferases; NADPH quinone:oxidoreductase; pharmacodynamics; pharmacokinetics; SNPs; sulfotransferases; XRCC1

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## 1. Introduction

The discovery of genetic polymorphisms in xenobiotic metabolizing enzymes stemmed from clinicians seeking reasons for the low tolerance of some patients to prescribed medications (Daly, 1995). Pharmacokinetic studies with probe drugs were able to demonstrate a metabolic basis for the increased toxicity and side effects, with genetic studies documenting underlying changes in genotype. Key early examples included the poor metabolizer phenotype for debrisoquine in relation to cytochrome P450 (CYP)2D6 polymorphism (Eichelbaum et al., 1979; Inaba et al., 1980), and the rapid versus slow acetylator phenotype brought about by polymorphism in *N*-acetyltransferase-2 (NAT2) (Hughes et al., 1954; Evans, 1989). The number of examples has spread across a wide range of xenobiotic metabolizing enzymes. The relevance to environmental toxicology is suggested by studies linking these metabolism polymorphisms to susceptibility for cancer and other adverse health outcomes related to workplace or environmental exposure (Sanderson et al., 2007; Wang and Huang, 2007; Nebert and Dalton, 2006). Such polymorphisms are a potential source of interindividual variability in internal dose of parent compound or active metabolite(s) and thus may affect the distribution of risk across the population. However, the application of polymorphism data to health assessment has been limited by the lack of genotype-phenotype information that can be incorporated into pharmacokinetic models predictive of internal dose.

Although metabolism genes have historically been the major focus, there is increasing awareness that genes involved in host response to chemical stressor (pharmacodynamics) can also be polymorphic (asthmatic response: Demchuk et al., 2007; DNA repair genes: Jiang et al., 2009; antioxidant defense: Bag and Bag, 2008; Ross and Siegel, 2004). This creates the possibility that xenobiotic metabolism and cellular defense genes can be polymorphic in the same individual, leading to the potential for a cumulative impact on biological response. The authors have previously characterized polymorphisms in a variety of metabolizing genes and through Monte Carlo simulation developed population distributions of enzyme activity in major ethnic groups (Ginsberg et al., 2009c). Table 1 is an overview of the previous effort combined with the enzymes addressed in the current analysis. These new analyses include several key conjugation enzymes

(glucuronidation, sulfation), along with enzymes involved in host defenses, in quenching epoxides (epoxide hydrolase), and in the regulation of redox status through quinone reduction (NADPH quinone oxidoreductase-1 [*NQO1*]). Because these enzyme systems contain numerous single-nucleotide polymorphisms (SNPs), the goal of the current effort was to select those SNPs with the greatest potential to affect internal dose and evaluate the feasibility of developing population distributions of enzyme activity.

The following sections review and analyze these polymorphic enzymes by providing background on enzyme function, genotype effect on phenotype, the frequency of influential alleles in the general population, and the pharmacokinetic and epidemiologic evidence that the polymorphisms can alter xenobiotic fate and susceptibility to human disease. Literature searches on PubMed and TOXLINE were the main sources of bibliographic information. Accession numbers for SNPs were obtained from the primary research articles when available. However, in numerous cases, they were only available from online databases that catalogue genes and their variants: <http://www.genecards.org/>; <http://www.ncbi.nlm.nih.gov/snp>; <http://www.pharmgkb.org/>; <http://alfred.med.yale.edu/>. These databases also provided useful allele frequency information in certain cases.

Although the terms pharmacokinetic and toxicokinetic are often used interchangeably, they generally refer to the body's handling of pharmaceuticals on the one hand or environmental toxicants on the other. Toxicokinetics can also be used to describe high-dose pharmaceutical behavior. This paper refers to the kinetics for both types of agents and rather than switching terms back and forth, the terms pharmacokinetic and pharmacodynamic are used consistently throughout. This is because much of the empirical polymorphism data stem from drug studies from which extrapolations can be made to the assessment of environmental chemicals.

## 2. Conjugation with sulfotransferases (SULTs)

SULTs are key contributors to the conjugation and removal of numerous phenolic xenobiotics and a variety of endogenous compounds, including neurotransmitters and steroid hormones. Hydroxylated metabolites are rendered less active and more water soluble via SULT-mediated conjugation with

**Table 1.** Overview of enzymes evaluated for potential impact of genetic polymorphisms on human health risk assessment.

Enzyme	Major polymorphisms	Distributional analysis <sup>1</sup>	Reference
Phase I			
CYP2D6	7 different SNPs contribute to poor metabolizer phenotype	Yes	Neafsey et al., 2009a
CYP2E1	5 different SNPs in regulatory sequences may alter gene expression	Not feasible	Neafsey et al., 2009b
Phase II			
GSTM1/T1/P1	Null polymorphisms in M1 and T1; 3 coding region SNPs in P1 alter activity in substrate-dependent manner	Yes	Ginsberg et al., 2009c
NAT1/NAT2	5 different SNPs in NAT2 contribute to slow acetylator phenotype	Yes	Walker et al., 2009
SULT (6 isozymes)	SULT1A1*2 associated with 2–10-fold less activity	Feasible	This paper, Section 2
UGT	4 enzymes with influential SNPs	Feasible	This paper, Section 3
Detoxification			
ALDH2	*2: null activity	Yes	Ginsberg et al., 2002a
EPHX1	Tyr113His—small effect His139Arg—small effect	Not feasible	This paper, Section 4
NQO1	*2: null activity	Feasible	This paper, Section 5
PON1	1 coding region SNP alters activity in substrate-dependent manner; 2 regulatory sequence SNPs decrease gene expression	Yes	Ginsberg et al., 2009a
XRCC1	Arg399Gln: 4-fold decrease in DNA repair function	Feasible	Manuscript in preparation

<sup>1</sup>Yes response in this column indicates that a distributional analysis was feasible and actually conducted as reported in the cited paper. Feasible means that a distributional analysis was judged possible based upon polymorphism effect on enzyme activity, substantial frequency of polymorphisms (>5%), and sufficient data for Monte Carlo analysis. In these cases a full distributional analysis has not yet been conducted. Not feasible means that the database is lacking in one or more areas.

the sulfate anion. SULTs are also involved in the conjugation of estradiol and related estrogenic structures, the breast cancer drug tamoxifen, and other hormonally active substrates such as the estrogen/testosterone precursor dehydroepiandrosterone (DHEA). Therefore, SULTs play an important role in xenobiotic metabolism and hormonal balance. Sulfation typically decreases biologic activity but in certain cases can increase toxicity or pharmacological activity. Sulfate conjugates can also serve as a pool of inactivated metabolite in the systemic circulation that can be reactivated by tissue sulfatases.

SNPs have been found at several hundred locations across the SULT gene family, with the majority occurring in noncoding regions (Hildebrandt et al., 2007). Only a limited number of SULT SNPs are known to modulate enzyme activity and many of these occur at low frequency in the populations studied. The most extensively studied SNP is *SULT1A1*\*2 (rs9282861, chromosome 16:28525015) because of its relatively high frequency and evidence for effect on enzyme activity.

### 2.1. SULT properties and function

The SULT super family of cytosolic enzymes encompasses 13 genes organized into four families based upon amino acid homology and chromosomal location (Lindsay et al., 2008). SULT enzymes can also be membrane-bound, but the cytosolic enzymes are considered of greater relevance to risk assessment because of their ability to conjugate a wide range of xenobiotics and endogenous hormones (Nowell and Falany, 2006).

Cytosolic SULT enzymes have been detected in a wide range of human tissues, including liver, lung, kidney, brain, gastrointestinal tract, skin, breast, and platelets (Lindsay et al., 2008). Due to the presence of the major isoform,

*SULT1A1*, in platelets, this has been utilized as a convenient biological specimen for phenotyping human populations (Ohtake et al., 2006; Weinshilboum, 1990). SULTs mediate the transfer of the sulfonyl group ( $\text{SO}_3^-$ ) from the sulfate donor 3'-phosphoadenosine-5'-phosphosulfate (PAPS) to hydroxyl, sulfhydryl, amino, or *N*-oxide moieties of acceptor molecules. The availability of the cofactor, PAPS, can be limiting, as it is a depletable pool whose synthesis depends upon the availability of inorganic sulfate from the circulation (Lindsay et al., 2008; Wang et al., 1998; Forbes-Bamforth and Coughtrie, 1994). The overall reaction requires binding of PAPS and substrate to SULT with the release of sulfonated product and 3'-phosphoadenosine-5'-phosphate.

#### 2.1.1. Endogenous and exogenous substrates

Table 2 summarizes major SULT enzymes for which substrate preference, biochemical role, and SNPs have been explored. The *SULT1A* family is generically known as phenolic SULTs because of their ability to sulfonate the hydroxyl moiety of a wide variety of phenols. It has four members (SULTs 1A1, 1A2, 1A3, 1A4), with *SULT1A1* being the quantitatively predominant *SULT1A* member in human liver. *SULT1A1* has broad spectrum activity, with at least some activity against most substrates that are candidates for sulfonation. However, functional specialization has been described in which *SULT1A3* appears to be more capable of conjugating catecholamine neurotransmitters, *SULT1E1* has specificity for catechol estrogen metabolites, *SULT1C1/1C2* has considerable activity with regards to *N*-hydroxyarylamines procarcinogen activation, and *SULT2A1* appears most active towards DHEA and other hydroxysteroids.

Many pharmacologic agents and environmental chemicals undergo oxidative (often referred to as Phase I) metabolism in which cytochrome P450 creates an oxidized metabolite.

**Table 2.** Overview of major cytosolic SULT enzymes.<sup>1</sup>

Enzyme	Chromosome	Substrates	SNPs
1A1	16	Broad spectrum, able to conjugate phenolic compounds including model substrates 4-nitrophenol, 2-naphthol; drugs (paracetamol, hydroxytamoxifen, minoxidil), aromatic and heterocyclic amines (e.g., <i>N</i> -hydroxy-PhIP), hormones (estradiol, diethylstilbestrol, iodothyronine); also conjugates catechol neurotransmitters (dopamine, epinephrine).	14 SNPs in coding region, most common 1A1*2 at 30%
1A2	16	Broad spectrum, 2-naphthol, minoxidil, 4-nitrophenol, various promutagens; expression levels low in liver and other tissues	5 SNPs, *2 and *3 most common at 18 and 29%
1A3	16	Primarily associated with catecholamine metabolism, expressed in brain; also conjugates other phenolic substrates	4 SNPs, all at less than 5%
1E1	4	High affinity for 17 $\beta$ -estradiol and related estrogens and their metabolites	3 SNPs, all at less than 1%
1C2	2	Activation of <i>N</i> -hydroxyarylamines	3 SNPs, *2 most common at 6.7%
2A1	19	Hydroxysteroids including DHEA, androgens, pregnenolone, bile acids	4 SNPs ranging from 3–13%

<sup>1</sup>Data from Lindsay et al., 2008, and Hildebrandt et al., 2007.

Although more water soluble, the oxidized metabolite may retain pharmacological activity (e.g., tamoxifen, carbamazepine) or may be made more toxic (e.g., benzene, aromatic amines). SULT-mediated conjugation is a critical line of defense, involved in the clearance of many hydroxylated metabolites. SULTs generally have a low  $K_M$ , which makes them an important first option for metabolic transformation for relatively low levels of exogenous or endogenous phenols. Cytosolic sulfate pools are limited and can be depleted at higher chemical doses, leading to a switchover to other conjugation systems. In many cases, SULTs share xenobiotic substrate preferences with the UDP glucuronyltransferases (UGTs), which generally have higher  $V_{max}$ , and also higher  $K_M$ , as exemplified with acetaminophen (Gelotte et al., 2007).

Benzene is an example of an environmental toxicant whose phenolic metabolites are substrates for SULTs as shown *in vitro* (Orzechowski et al., 1995) and in monkey pharmacokinetic studies (Sabourin et al., 1992). However, a molecular epidemiology study in 152 benzene-poisoned Chinese workers and 152 controls failed to find an influence of *SULT1A1* polymorphism on susceptibility to benzene toxicity (Gu et al., 2007). This is not surprising given the numerous exposure, pharmacokinetic (e.g., UGTs), and pharmacodynamic variables that can modulate benzene risks *in vivo*. Other environmental agents can be metabolized via conjugation with SULTs. A study of workers exposed to a mixture of dinitrotoluenes measured a variety of biomarkers, including urinary metabolites, hemoglobin adducts, and chromosomal aberrations (Sabbioni et al., 2006). Low-activity forms of *SULT1A1* and *SULT1A2* were associated with lower levels of hemoglobin adducts and chromosomal damage. Although contrary to expected results, this may indicate sulfate stabilization of the metabolites, which could then be made available to blood cells and tissues by sulfatases. A variety of other phenolic compounds of environmental interest, including polycyclic aromatic hydrocarbon metabolites and phenols in creosote, have been found to be substrates for sulfate and glucuronide metabolism (Ogata et al., 1995; Lee et al., 2007).

Although typically a step in the chain of events leading to chemical detoxification, SULT-mediated conjugation

is in some cases an activation step. These cases include a variety of aromatic amines (e.g., 4-aminoazobenzene, *N*-hydroxy-2-acetylaminofluorene, safrole, 2,3-estragole, and 6-hydroxymethylbenzo(a)pyrene (Glatt, 2000)). When these carcinogens become conjugated by SULT, the *O*-sulfated product is unstable, leading to spontaneous removal of sulfate anion and leaving behind an electrophilic, positively charged carbonium ion. Transfer of individual SULT genes into the Ames *Salmonella* tester strains enabled the comparison of mutagen activation across enzymes (Meinl et al., 2002). The SULT-modified tester strains can readily activate promutagens with over 100 such compounds activated in this manner (Glatt et al., 2000). Incorporation of *SULT1A1* and *SULT1A2* tended to have the greatest activation activity for the three promutagens tested in Meinl et al. (2002): 1-hydroxymethylpyrene (1-HMP), 2-hydroxylamino-5-phenylpyridine (OH-APP), and *N*-hydroxy-2-acetylaminofluorene (OH-AAF).

SULT-mediated conjugation is also essential to the metabolism of a wide variety of endogenous and xenobiotic substrates that contain the catechol structure, including neurotransmitters (adrenergic transmitters), steroid hormones (estrogen metabolites), dietary constituents (flavonoids, coumarins), and a variety of pharmacologic agents (carbidopa, apomorphine). Although glucuronidation and catechol-*O*-methyl transferase (COMT) can also conjugate catechols, SULTs appear to be of particular importance given that much circulating catecholamine, as exemplified by administered levodopa, is in the sulfated form (Mizutani et al., 1995). Testing of human recombinant SULT enzymes allowed for comparison of catechol conjugation rates across a wide range of enzymes and substrates (Taskinen et al., 2003). SULTs 1A1, 1A2, 1A3, and 1B1 were capable of conjugating all of the tested substrates. The “catecholamine SULT,” *SULT1A3*, was among the highest activity enzymes, although *SULT1A1* had comparable or greater activity in numerous cases.

Sulfonation is a key step in the regulation and distribution of a variety of steroid hormones, as the sulfated form is inactive but can be transported to other tissues where sulfatases regenerate the active hormone (Hansen et al., 2004). Circulating levels of the conjugated form of the steroid can be many times greater than the parent compound, as exemplified

by dehydroepiandrosterone (DHEA) (Hinson et al., 2003; Sato et al., 2008). Pregnenolone is an example where sulfonation leads to a conjugate with biological activity, neuromodulation, which is unrelated to the parent compound (Gibbs et al., 2006); this activity is terminated by sulfatases. Whereas *SULT2A1* is most active towards DHEA and pregnenolone, *SULT1E1*, sometimes referred to as estrogen sulfotransferase, has a particularly low  $K_M$  for estrogens and their catechol metabolites and so is important at physiological levels of these hormones (Hui et al., 2008). The oxidized metabolites of estrogen can be precursors to highly reactive quinones capable of DNA damage. Conjugation of catechol estrogens via *SULT1E1* represents an important detoxification pathway (Raftogianis et al., 2000).

### 2.1.2. Measurement of SULT activity

Populations have been phenotyped for *SULT1A1* activity by isolation of platelets from blood and incubating platelet cytosol with the cofactor PAPS and a variety of different substrates. The most commonly used substrate, 4-nitrophenol, is not unique to *SULT1A1*, as it is also a substrate for *SULT1B1* (Tabrett and Coughtrie, 2003; Raftogianis et al., 1999). 4-Hydroxytamoxifen has been used to probe for *SULT1A1* in platelets from a Japanese population because this active tamoxifen metabolite is an excellent though not necessarily specific substrate for *SULT1A1* (Ohtake et al., 2006). Other SULT subfamilies have a range of activity that can be overlapping with *SULT1A1*, yet distinctions have been found (Table 2). Dopamine at low incubation concentration has been found to be a specific probe for *SULT1A3*, with hydroxydopamine and hydroxyserotonin also specific for this SULT (Yasuda et al., 2007). For *SULT1E1*, 17 $\beta$ -estradiol at nanomolar concentration is a suitable probe substrate (Zhang et al., 1998). In addition to platelets, human liver bank samples have been used to evaluate interindividual variation in *SULT1A1* activity (Tabrett and Coughtrie, 2003; Rossi et al., 2004). For other SULT enzymes, platelets are not as useful an indication

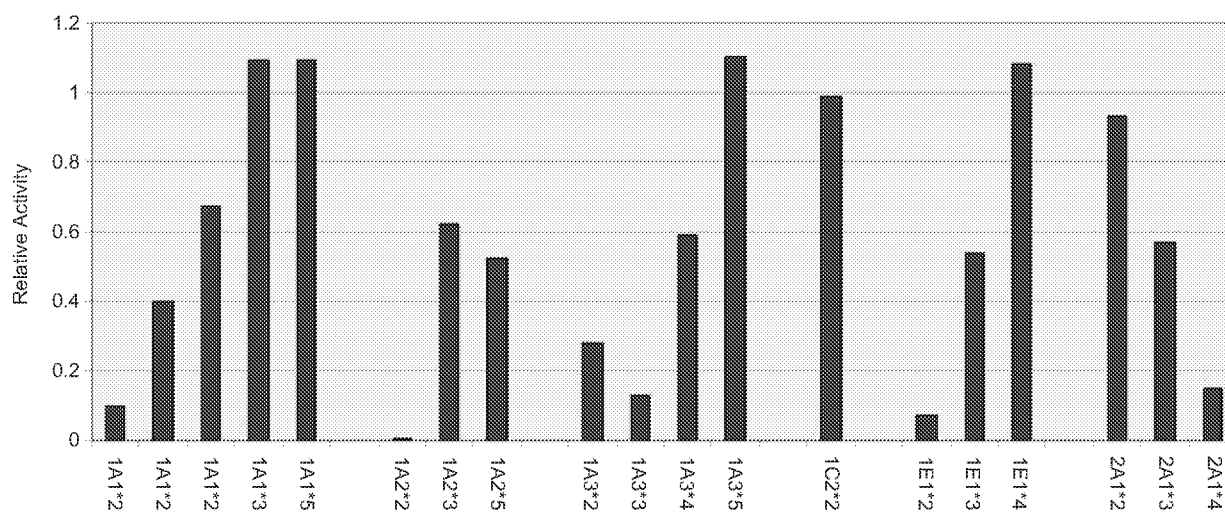
of enzyme activity. Genotype/phenotype relationships for these SNPs have been studied via *in vitro* expression systems rather than in population studies.

### 2.2. Effect of SNPs on enzyme activity

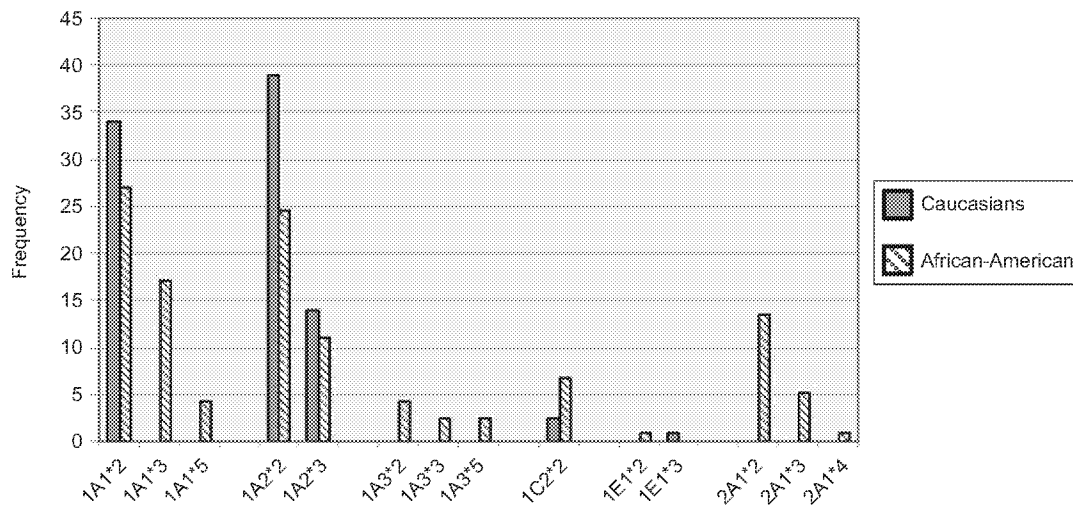
Genetic polymorphisms have been evaluated most extensively for *SULT1A1* due to its broad substrate specificity and widespread tissue distribution. Although 14 coding region SNPs have been identified, most are rare. *SULT1A1*\*2 is the only one considered common, as it occurs in approximately 30% of Caucasians and African Americans and in 10% of Asians. Table 2 shows that other SULT genes are polymorphic, with allele frequencies relatively high for *SULT1A* family variants but considerably lower for *SULT1E1*, *SULT1C2*, and *SULT2A1*. Additional detail is provided in Figures 1 and 2, which present SNP effects on enzyme activity and allele frequencies, respectively. These results are summarized from publications that have catalogued the pharmacogenetics of the SULT superfamily (Lindsay et al., 2008; Hildebrandt et al., 2007; Nowell and Falany, 2006) as well as the online cataloguing databases cited above.

#### 2.2.1. *SULT1A1* SNPs

The most widely studied SNP is found in *SULT1A1* in which a coding region polymorphism involving G to A transition occurs at nucleotide 638 (Jones et al., 1995; Raftogianis et al., 1997), although more recent analyses of the primary transcript (NM\_001055.2) indicate that this SNP occurs at nucleotide 748 of the mRNA (NLM database at <http://www.ncbi.nlm.nih.gov/>). This SNP causes arginine to be replaced by histidine at amino acid 213, forming the *SULT1A1*\*2 variant (rs 9282861, chromosome16:28525015). The result is an enzyme with no apparent change in catalytic activity but having lower enzyme stability. The recombinant *SULT1A1.2* gene product was associated with lower protein levels (54%) and less activity (67%) relative to the reference



**Figure 1.** Activity of SULT variants relative to the reference genotype. Several results are presented for *SULT1A2.2* due to the wide range reported in the literature as described in the text. Results for other enzymes are either from only a single source or are more consistent.



**Figure 2.** Allele frequency for SULT polymorphisms (from Lindsay et al., 2008; Hildebrandt et al., 2007; Meinel et al., 2002, and online databases described in text).

(sometimes referred to as wild-type) gene (Hildebrandt et al., 2007) when assayed with 4-nitrophenol as substrate. In another genotype/phenotype study, *SULT1A1* variants were expressed in several different recombinant systems and tested against a battery of sulfotransferase substrates including 4-nitrophenol, 4-hydroxytamoxifen, quercetin, chrysin, estradiol, and hydroxyestradiol (Nagar et al., 2006). The  $V_{\max}$  for the *SULT1A1.2* product was consistently 1 to 2 orders of magnitude below the reference enzyme, with this attributed to the 6-fold shorter half-life of the variant protein. A study of 103 Japanese subjects from whom platelets were isolated and incubated with the *SULT1A1* probe 4-hydroxytamoxifen found considerable interindividual variability. Individuals who were *SULT1A1\*2* homozygotes had lower activity ( $124 \pm 66$  units,  $N=4$ ) than those with the reference genotype ( $303 \pm 267$  units,  $N=73$ ) (Ohtake et al., 2006). An earlier study using 4-nitrophenol as substrate in platelets from 33 subjects found *SULT1A1\*2* homozygotes to have 7.7-fold lower activity than the reference population (Raftogianis et al., 1997). Also in platelets, a study of *SULT1A1* activity from 279 subjects found homozygous variants to have 53% of the reference activity when tested against 2-naphthol (Nowell et al., 2000). These investigators also found recombinant *SULT1A1\*2* to have only 10–25% the activity of the reference gene in conjugating *N*-hydroxyaminobiphenyl (*N*-OH-ABP) or 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (*N*-OH-PhIP) to DNA reactive metabolites (Nowell et al., 2000).

Although the *SULT1A1\*2* allele generally has shown activity that is 10–67% of the reference gene (Figure 1), the differential is smallest when this is studied in human liver samples (Rossi et al., 2004). This may be a function of the presence of other SULT enzymes that can also metabolize the substrate used, 4-nitrophenol.

The *SULT1A1\*3* variant has a coding region SNP at amino acid 223 (methionine to valine) (rs1801030, chromosome16:28524986). Its  $V_{\max}$  was consistently below the reference protein but above that for *SULT1A1.2* in a variety of test systems, although  $V_{\max}/K_M$  ratios were similar

and sometimes even higher than the reference enzyme for the *SULT1A1.3* variant (Nagar et al., 2006). Hildebrandt et al. (2007) report this variant and the *SULT1A1.5* variant (Phe247Leu) (rs28374453, chromosome16:28524914) to have similar activity as the reference gene product in their expression system using 4-nitrophenol as substrate (Figure 1).

## 2.2.2. SNPs in other SULTs

SNPs have also been identified in a number of other SULT enzymes. Recombinant systems using preferred substrates have identified activity differences between variant and reference enzymes in a number of cases, as summarized in Figure 1. *SULT1A2* variants have implications for enzyme activity, with one in particular, *SULT1A2\*2*, yielding a gene product with a very low  $V_{\max}/K_M$  ratio. This allele contains two SNPs, Ile7Thr (rs4149404, chromosome 16:28514733) and Asn235Thr (rs1059491, chromosome 16:28511156). More modest declines in enzyme activity are associated with *SULT1A2\*3* (Pro19Leu) (rs10797300, chromosome 16:28514697). The significance of this enzyme (*SULT1A2*) is unclear, as liver bank studies have failed to find an impact of the *SULT1A2\*2* allele (Rossi et al., 2004). This may be because *SULT1A2* has been detected at only low levels of expression in human liver and appears to have a splicing defect that prevents its translation into protein (Ozawa et al., 1998; Meinel et al., 2002; Nowell et al., 2005). The *SULT1A3* gene appears in two copies on the short arm of chromosome 16, with a nomenclature that distinguishes between these identical genes (*SULT1A3/1A4*) (Hildebrandt et al., 2004). In combination, the two genes have four coding region SNPs that alter amino acid sequence with these SNPs detected in African American but not Caucasian subjects (Hildebrandt et al., 2004, 2007). The *SULT1A3/1A4\*2* allele (Lys234Asn; chromosome 16:30122434), *SULT1A3/1A4\*3* allele (Pro101Leu), *SULT1A3/1A4\*4* allele (Pro101His), and *SULT1A3/1A4\*5* allele (Arg144Cys) are all relatively infrequent (<1% to 4%), with three of the four associated with decreased enzyme

activity (sulfonation of dopamine) and protein levels in recombinant expression systems (Hildebrandt et al., 2004, 2007). The *SULT1A3/1A4*\*2 and *SULT1A3/1A4*\*3 variants were associated with particularly low protein levels and enzyme activity (Figure 1).

*SULT1C2* has four SNPs that affect amino acid content, with three of the four affecting enzyme activity. *SULT1C2*\*3 (Asp60Ala; rs72549391, chromosome 2:108277144), *SULT1C2*\*4 (Arg73Gln; rs17036058, chromosome 2:108277183), and *SULT1C2*\*5 (Ser111Phe; rs72549392, chromosome 2:108283780) are associated with 85% or more decrease in enzyme activity. However these variants have frequencies of 1% or less in Caucasians and African Americans and so are not presented in Figure 1. The only variant with >1% occurrence (*SULT1C2*\*2, Ser255Thr) (rs17036104, chromosome 2:108288468) has similar activity as the reference gene (Freimuth et al., 2001; Hildebrandt et al., 2007). Several *SULT1E1* variants have been found with *SULT1E1*\*2 (Asp22Tyr; rs11569705, chromosome 4:70757888), yielding a protein with less than 10% of the reference enzyme activity. Two other SNPs, *SULT1E1*\*3 (Ala32Val; rs11569703) and *SULT1E1*\*4 (Pro253His; rs11569712, chromosome 4:70744482), are associated with 50–100% of reference activity and these variants are present in 1% or less of the population (Adjei et al., 2003; Hildebrandt et al., 2007).

*SULT2A1* has three coding region SNPs, all of which have been found in African Americans but not Caucasians (Thomae et al., 2002; Wilborn et al., 2005). Recombinant forms of the three variants were expressed in COS-1 cells, with the more common variant (*SULT2A1*\*2, Ala261Thr; rs11569679, chromosome 19:53066601) associated with conjugation activity that was 93% of reference activity. The less common variant, *SULT2A1*\*3 (Ala61Pro; rs11569681, chromosome 19:53078804), was associated with 57% of reference activity, whereas the rare variant, *SULT2A1*\*4 (Lys227Glu; rs11569680, chromosome 19:53069792), was at 15%. These decreases corresponded to similar decreases in protein content, suggesting that either diminished gene expression or protein stability was responsible for the lower activity seen in these variants (Thomae et al., 2002).

### 2.3. Frequency of SULT variants

Figure 2 summarizes allele frequency results across a variety of the most studied SULTs in Caucasians and African Americans. Data in Asian populations are more limited (Lindsay et al., 2008; Ohtake et al., 2006). *SULT1A1*\*2 is commonly found in Caucasian (34%), African American (27%), and Asian (8–17%) populations. In contrast, other *SULT1A1* polymorphisms were not found in Caucasians but were present at 4–17% in African Americans. The *SULT1A2*\*2 haplotype is present at relatively high frequency in Caucasians and African Americans (25–40%), with the *SULT1A2*\*3 allele somewhat less frequent (10–15%) (Carlini et al., 2001; Raftogianis et al., 1999; Lindsay et al., 2008). *SULT1A2*\*5 appears to be rare. SNPs in other members of the SULT family appear to be infrequent in Caucasians, but this is not necessarily the case for other groups, as African Americans have *SULT1A3*

SNP frequencies of 2.5–4%, *SULT1C2* SNP frequency of 7%, and *SULT2A1* variants at 1–14% (Figure 2). *SULT1E1* variants occur at low frequency in Caucasians and African Americans, with frequencies generally less than 1% when the variants are encountered (Hildebrandt et al., 2007).

### 2.4. Other sources of variability in SULT activity

Studies examining the extent of variation in SULT activity in relation to identified SNPs have found that relatively little (approximately 10%) of the variance seen in human liver can be explained by genotype (Rossi et al., 2004; Tabrett and Coughtrie, 2003). This could be due to other SNPs not yet identified or to physiologic, dietary, or pharmacologic factors that modulate SULT enzyme levels. Further, the number of copies of the gene can be variable as seen in particular for *SULT1A1* (Hebbring et al., 2006).

SULTs are inducible by classical microsomal enzyme inducers, as demonstrated in human and monkey liver cell cultures (Nishimura et al., 2008). When incubated with the inducers rifampicin, dexamethasone, and omeprazole, there was a dose-dependent increase in *SULT2A1* but not *SULT1A1* enzymes. Glucocorticoids were shown to be inducers of *SULT1A3* but not *SULT1A1* in human HepG2 cells (Bian et al., 2007) and of *SULT2A1* in humans and mice (Gamage et al., 2006). In a broader study in mouse liver examining 11 different SULT enzymes and 15 different microsomal enzyme inducers, induction was found to vary across treatments, with aryl hydrocarbon (AH) receptor ligands suppressing SULT levels, peroxisome proliferator ligands having little effect in males but suppressing several isoforms in females, and other inducers specifically enhancing *SULT1E1* activity (e.g., pregnane X receptor ligands) (Alnouti and Klaassen, 2008). Consistent with this is the finding that pyrene induction of *SULT1A1* is independent of the AH receptor, as shown in AH-nonresponsive mice (Lee et al., 2007). Instead pyrene's induction appears to be mediated by the constitutive androstane receptor (CAR).

Dietary components may alter SULT expression, as human ingestion of brussels sprouts has been shown to decrease *SULT1A1* and *SULT1A3* protein levels and enzyme activities. These data come from lymphocytes from seven subjects (Hoelzl et al., 2008). It is not known whether this effect occurs at the level of gene expression or altered protein stability. Coffee has chemoprotective diterpenes (kawheal and cafestrol) that are hypothesized to modulate a variety of hepatic enzymes. Rat ingestion of coffee had no effect on SULT levels whereas administration of the diterpenes decreased SULTs by 25% (Huber et al., 2008). Other drugs and dietary components have been found to decrease *SULT1A1* levels in recombinant systems, including mefenamic acid, salicylic acid, and quercetin (Pacifi, 2004).

SULT activity measured in platelets has been shown to be gender and season dependent, with greater seasonal fluctuation (summer peak) seen in females than males (Marazzati et al., 1998). A study of platelet *SULT1A1* activity in 279 subjects found males to be consistently lower than females across genotypes (68–92% of female levels) (Nowell et al.,

2000). Expression of other SULTs may be gender dependent, as *SULT1E1* levels in mice were testosterone dependent, with levels in various adipose tissues greater in males and ablated by castration (Khor et al., 2008). *SULT1E1* was also expressed in testis but not other tissues in either gender, and treatment of females with testosterone increased their adipose tissue levels of this SULT. This evidence is consistent with *SULT1E1*'s role in regulating circulating estrogen levels and with adipose tissue being a major locus of gender-specific estrogen effects. For example, male *SULT1E1* knockout mice exhibited increased adipose accumulation due to decreased removal of estrogen from these tissues (Khor et al., 2008).

An additional source of phenotypic variability is gene copy number, as probing of the DNA from 362 Caucasian and 99 African American samples found multiple copies of the *SULT1A1* gene to be a commonplace occurrence (Hebbring et al., 2006). Three or more gene copies occurred 26% of the time in Caucasians and 63% of the time in African Americans. Evaluation of the source of variability in *SULT1A1* activity in 267 liver bank samples and 23 platelet samples found that the low-activity *SULT1A1*\*2 allele (Arg213His) and two upstream variants did not have a statistically significant effect on activity beyond that already caused by copy number variation, which had a very pronounced effect (Hebbring et al., 2006). These data suggest that gene copy number is an important source of variability in *SULT1A1* activity across the population and should be probed in addition to individual SNPs.

#### 2.4.1. Ontogeny of SULTs

SULT expression occurs early in gestation with relatively high levels of *SULT1A1* and *SULT1A3* detected in fetal livers sampled from a human tissue bank ( $N=44$  livers) (Richard et al., 2001). Postnatal *SULT1A1* levels remained at the prenatal levels whereas *SULT1A3* levels dropped precipitously in the postnatal samples. *SULT1A1* levels continued to rise postnatally to become approximately 2 times higher in adult as compared to fetal liver. In contrast, *SULT1A3* is virtually absent from adult liver (Richard et al., 2001). Similar results were found in a larger study of perinatal human liver samples ( $N=235$ ) in which prenatal and postnatal *SULT1A1* levels were similar whereas *SULT2A1* showed an increasing content throughout gestation and into postnatal life (Duanmu et al., 2006). *SULT1E1* showed the opposite trend, with hepatic content decreasing from the first trimester onward into postnatal life. The fact that the greatest *SULT1E1* content was in first trimester males suggests that this enzyme is important to development of the male phenotype in utero. This is consistent with *SULT1E1*'s role as a key estrogen-conjugating enzyme (Duanmu et al., 2006).

The SULT developmental profile, with activity readily detectable in the perinatal period, has clinical implications as seen in accidental childhood poisoning by acetaminophen. A pharmacokinetic study evaluated the clearance of a 10 mg/kg dose in neonates (0–2 days old), young children (3–9 years old), older children (12 years old), and adults (Miller et al., 1976). There was a similar rate constant for acetaminophen metabolic clearance across groups. However, the urinary profile showed

a greater proportion of sulfate metabolism in the younger age groups, which switched to predominantly glucuronide formation in 12-year-olds and adults. Similar results were found for salicylamide in which a 5 mg/kg dose was excreted predominantly as the sulfate conjugate in 7–10-year-olds but as the glucuronide in adults (Alam et al., 1977). These studies suggest that SULT-mediated conjugation may play a larger role in xenobiotic detoxification in children than in adults.

#### 2.5. Evidence of SULT modulation of drug clearance or effectiveness

SULT enzymes rarely are alone in conjugating substrates, with SULT isoforms generally having broad substrate specificity and overlap in function with each other and with other enzyme families. Most notably, the glucuronidation system can conjugate many SULT substrates. Therefore, evidence of SULT modulating drug clearance or effectiveness would be instructive in showing the *in vivo* importance in changes in SULT activity. However, studies with tamoxifen (Gjerde et al., 2008; Nowell et al., 2002, 2005; Wegman et al., 2005, 2007) and levodopa (Dousa et al., 2003) failed to find a substantial pharmacokinetic influence of *SULT1A1* or *SULT1A3* polymorphism, suggesting that other pathways *in vivo* can compensate for the effect of the polymorphism on conjugation. The evidence that gene copy number may be more influential than individual SNPs may also explain the lack of SNP effect on drug clearance (Hebbring et al., 2006).

#### 2.6. Epidemiological associations with SULT polymorphism

Several studies have examined SULT alleles in relation to a variety of health outcomes, most commonly cancer. Hormonally responsive tumors have been a major focus because of the role of *SULT1A1* and *SULT1E1* in conjugating estrogen. Perhaps the strongest link between genotype and increased cancer risk is with endometrial cancer. A study of 150 endometrial cancer patients and 165 controls found that the presence of *SULT1A1*\*2 conferred a 2–3-fold greater risk relative to those with the reference genotype (Hirata et al., 2008). This finding is supported by others also showing an association between the variant (lower-activity) *SULT1A1* allele and endometrial cancer (Mikhailova et al., 2006). In a study evaluating estrogen replacement therapy as a causative factor in endometrial cancer, impaired sulfation as represented by *SULT1A1*\*2 was found to be a contributing factor to the increased risk (Rebeck et al., 2006). However, a recent study of breast, ovarian, and endometrial cancers in Russia failed to find a link to *SULT1A1* polymorphism. The one exception was a small but statistically significant protective effect of this polymorphism for ovarian cancer (Gulyaeva et al., 2008).

Breast cancer incidence in relation to SULT polymorphism has been studied with overall inconsistent results. An evaluation of 989 breast cancer cases in South Korea failed to find an association with *SULT1A1* polymorphism and found weak and inconsistent associations for several *SULT1E1* polymorphisms that do not change protein structure (intronic or upstream

SNPs) and that lack genotype/phenotype data (Choi et al., 2005). Similarly, a study of 444 breast cancer cases and 227 controls failed to find an association between *SULT1A1*\*2 and breast cancer risk (Seth et al., 2000). However, several similarly powered studies have shown such an association (Zheng et al., 2001; Han et al., 2004). These disparate findings may be related to different risk factors and typology of the breast cancers prevalent in the various populations studied.

The *SULT1A1*\*2 allele has also been shown to be linked to lung cancer, as a study of 463 Caucasian lung cancer cases showed an overall 1.4-fold increased risk in those with the SNP (Wang et al., 2002). This risk was greater in women than men and was accentuated in smokers. In contrast, a study of 384 bladder cancer cases showed an apparent protective effect of the *SULT1A1*\*2 allele, especially in women (odds ratio = 0.42) (Zheng et al., 2003). This association was seen in smokers and nonsmokers alike. Gastric cancer may also be modulated by SULT status, as the variant *SULT1A1*\*2 allele was associated with a 3.3-fold higher risk in homozygous variants (Boccia et al., 2005). This association was more pronounced in males and in those consuming alcohol, char-broiled meats, and tobacco.

## 2.7. Summary: Risk implications of SULT polymorphism and potential for distributional analysis

### 2.7.1. Is the enzyme important to toxicant action?

Although SULTs are effective in conjugating a variety of biologically active substrates, they are not the only conjugation system. In particular, UGTs have similar hydroxyl and phenolic substrates, with the tendency for glucuronidation to be more important at high substrate concentration and sulfonation more important at low concentration. There is only limited evidence that *SULT1A1* polymorphism can affect the *in vivo* clearance of pharmacologic agents, as alternative clearance pathways, gene copy number, and the small number of studies creates uncertainty in this area. Due to differences in ontogeny, sulfation appears to predominate over glucuronidation in young children. Therefore, SULT polymorphism and variability in *SULT1A1* copy number may have the greatest implications for human health risk for (1) substrates lacking alternative enzyme systems to compensate for SULT deficiency in a particular tissue; (2) chronic low-dose exposure due to the tendency for SULT to have a lower  $K_M$  than glucuronidation enzymes; and (3) early life, due to the slower appearance of glucuronidation capacity.

Molecular epidemiology evidence suggests that *SULT1A1* polymorphism may modulate risk in certain hormonally mediated cancers (e.g., endometrial), and possibly also in gastric and smoking-related cancers. This suggests that SULTs can play a key role in the disposition of estrogen and other etiologic agents.

### 2.7.2. Are there influential SNPs of sufficient frequency to have a broad impact?

As depicted in Figure 1, a number of SULT SNPs have been identified that decrease enzyme activity, typically by decreasing protein stability. The most heavily studied SNP

is *SULT1A1*\*2, as it is expressed in many tissues, has broad substrate specificity, and is relatively common across a variety of ethnic groups (approximately 30% allele frequency in Caucasians and African Americans, somewhat lower in Asians). This allele is associated with decreased SULT activity against numerous conjugation substrates in both recombinant expression systems and in liver and platelet samples from human populations. A range of results has been found in genotype/phenotype studies, from less than a 2-fold decrease in conjugation activity to more than a 10-fold decrease.

There are a number of other SULTs with polymorphisms that can affect function, as depicted in Figure 1. However, these SNPs are less well studied, as the data come from recombinant systems in which there is no indication of phenotypic variability in the population. Further, most of these SNPs are of low or rare frequency. The major exception is *SULT1A2*\*2 in that it has functional significance and is present at 25–38% in Caucasians and African Americans (Figure 2). Thus, this variant also merits consideration as a source of variability in risk assessments of SULT substrates. Although there is a substantial effect of *SULT1A3*\*2 and *SULT1A3*\*3 variants on enzyme activity, their allele frequencies are low (less than 5%). Similarly, *SULT1E1*\*2 and *SULT2A1*\*4 are SNPs with a large influence on enzyme activity but apparently of very low frequency. Overall, the evidence for functional significance and high frequency of *SULT1A1*\*2 and *SULT1A2*\*2 make these variants important to consider in health risk assessments of environmental agents.

### 2.7.3. Can the polymorphism data inform health risk assessments?

Useful data come from three population studies that associated the *SULT1A1*\*2 allele with decreased conjugation capacity in platelets (Ohtake et al., 2006; Raftogianis et al., 1997; Nowell et al., 2000). These studies are sizable ( $N=33$ –279) and examined a diversity of substrates (4-hydroxytamoxifen, hydroxylated aromatic amines, 2-naphthol, 4-nitrophenol). They reported a range of SNP effects (2–10-fold decrease in activity) and provide an indication of interindividual variability within genotype that is needed for Monte Carlo analysis of variability. When the frequency data available for *SULT1A1*\*2 are combined with genotype-phenotype information, a population distribution of *SULT1A1* activity can be developed as exemplified previously for aldehyde dehydrogenase-2 (Ginsberg et al., 2002a). However, it should be noted that these studies did not assess gene copy number. Evidence that the number of copies of *SULT1A1* is highly variable and can have a major influence on phenotype must also be taken into consideration as an additional source of genotypic variability (Hebbring et al., 2006).

The data are too limited to allow quantitative estimates of enzyme variability due to SNPs for the other SULT enzymes. Population studies of SNP effect on phenotype using appropriate tissues and substrates are needed to further explore the *in vivo* implications of *SULT1A2*\*2 given that it is associated with low enzyme activity *in vitro* and occurs at high

frequency. Additional evaluation of influential SNPs in *SULT1A3*, *SULT1E1*, and *SULT2A1* would also be useful.

#### 2.7.4. Outstanding questions regarding health risk implications of SULT polymorphisms

There are numerous factors that can affect phenotype in addition to the identified SNPs. Although some percentage of SULT variability may be explained by gene copy number variation (Hebbring et al., 2006), other host factors may also modulate the activity found including gender, ethnicity, age, medication use, diet, and the presence of SULTs with overlapping substrate specificity. In addition, glucuronidation and catechol-*O*-methyl-transferase (COMT) conjugation systems can, under certain circumstances, compensate for deficiency in SULT activity. There is only limited evidence of *in vivo* modulation of chemical fate by SULT polymorphism. Thus, although it is possible to construct a population distribution of *SULT1A1*\*2 activity, the implications of this distribution for health risk assessment will be uncertain without conducting a comprehensive pharmacokinetic analysis of the various interacting pathways.

Additional uncertainties have to do with the role of SULT in modulating toxicity. For some substrates, the conjugate is inactive and readily excreted, whereas for others the conjugate has an extended residence time and serves as a stable pool of metabolite that can be reactivated by tissue sulfatases. Finally, some substrates can be activated to more toxic forms via sulfation. Ultimately, the implications of SULT variability on human health risk assessment depend upon chemical-specific considerations.

### 3. Conjugation with uridine-diphosphate glucuronosyltransferase (UGT)

UGT enzymes are key contributors to the conjugation and elimination of numerous xenobiotics and endogenous compounds, including bilirubin and steroid hormones. UGTs primarily target hydroxyl or amino groups, increasing the compound's molecular weight and water solubility, thus facilitating fecal or urinary elimination. The importance of UGTs in metabolism and clearance has been demonstrated, as numerous therapeutic drugs or their metabolites are excreted primarily as the glucuronide metabolite (Maruo et al., 2005). Although typically considered a detoxification step, in some instances glucuronide conjugation can lead to chemical activation and toxicity (Burchell and Coughtrie, 1989). Further,  $\beta$ -glucuronidases present in the gastrointestinal tract and various tissues can remove the glucuronide moiety, leading to the local activation and recirculation of the de-conjugated metabolite.

#### 3.1. UGT properties and function

UGTs are membrane-bound enzymes located on the smooth endoplasmic reticulum of many tissues and across a phylogenetic spectrum that includes insects, fish, and mammals. Greatest UGT activity and cofactor (uridine diphosphoglucuronic acid [UDPGA]) content is typically in the liver, with

levels in extrahepatic tissues such as kidney, lung, and gastrointestinal tract approximately 10-fold lower (Cappiello et al., 1991). However, this is enzyme specific, as certain UGTs are expressed only in extrahepatic tissues (Strassburg et al., 1997, 1999).

UGTs mediate the transfer of a hydrophilic sugar moiety from UDPGA to hydroxyl-, amino-, carboxylic-, or sulfur-bearing constituents on the substrate. Catalysis depends upon numerous factors, including hydrophobicity of the substrate (Sorich et al., 2004), phosphorylation status of the enzyme (Basu et al., 2005), and cofactor availability. UDPGA availability is a rate-limiting factor and synthesis of this cofactor is governed by the availability of precursor glucose molecules (Banhegyi et al., 1988; Braun et al., 1997). Glycogen breakdown is the major source of glucose for this purpose and the rate of glycogenolysis is governed by cellular redox status as mediated via the reduced/oxidized glutathione ratio (GSH/GSSG). Decreases in this ratio or depletion of GSH favor glycogenolysis, which increases the supply of UDPGA and enhances glucuronidation of xenobiotics (Braun et al., 1997). The supply of UDPGA can be depleted by high conjugation demand, as documented by studies in which pretreatment with retinol or phenobarbital depleted UDPGA stores, leading to greater toxicity from subsequent acetaminophen administration (Bray and Rosengren, 2001; Doudar and Ahmed, 1987). UGT conjugation is typically a high- $V_{\max}$ , high- $K_M$  reaction, representing an important detoxification step for large amounts of substrate. Other conjugation reactions such as sulfation tend to predominate at lower doses, as documented with acetaminophen (Gelotte et al., 2007; Riches et al., 2009).

#### 3.1.1. UGT gene families

UGT enzymes are divided into two broad subfamilies, *UGT1* and *UGT2*, based upon sequence homology (Maruo et al., 2005; Nagar and Rummel, 2006). Although these UGT subfamilies appear on different chromosomes (chromosome 2 and 4, respectively) and have little structural similarity, the overlap in substrate specificity is still considerable. *UGT1* genes have four exons in common (exons 2–5) and are distinguished based upon variable sequences in exon 1. In contrast, *UGT2*s have six exons, all of which are different from *UGT1* exons, and all have variable sequences of their own. However, both subfamilies conjugate a variety of estrogens, androgens, pharmaceuticals, and environmentally relevant amines (e.g., benzidine) and phenols (see Table 3). Small planar phenols such as the analgesic drug acetaminophen tend to be metabolized most extensively by *UGT1A6* (Ciotti et al., 1997; Nagar et al., 2004). Bilirubin conjugation is an important UGT function that is attributed primarily to *UGT1A1*. Regarding other endogenous substrates, the *UGT1* subfamily tends to have greater activity towards estrogens and catechol estrogen metabolites, although *UGT2B7* is also important in this regard (Lepine et al., 2004) and the environmental estrogen bisphenol A is conjugated mainly by *UGT2B* enzymes in rats and humans (Hanioka et al., 2008; Yokota et al., 1999). The *UGT2* subfamily is generally

**Table 3.** UGT Isozymes: Substrates, polymorphisms, and identified alleles.

Enzyme	Substrates	SNP/amino acid change	Allele <sup>1</sup>
1A1	Bilirubin, estrogens ( $\beta$ -estradiol, hydroxyestradiols, hydroxyestrones), buprenorphine, flavonoids, anthraquinone, 4-nitrophenol	TATA insertion/none G211→A/Gly71Arg T1456→G/Tyr486Asp	UGT1A1*28 UGT1A1*6 UGT1A1*7
1A3	Estrone, hydroxyestrone, hydroxyestradiol, amines (cypheptadine, losartan), anthraquinones, femoprofen, ibuprofen, umbelliferone, flavonoids	T-66→C/none A-204→G/none A17→G/Q6R T31→C/W11R C133→T/R45W T140→C/V47A	None identified
1A4	Amines (clozapine, aminobiphenyl, naphthylamine, benzidine, aminofluorene, imipramine), 4-hydroxytamoxifen, 5 $\alpha$ -pregnene-3 $\alpha$ ,20 $\beta$ -diol	C70→A/Pro24Thr	None identified
1A6	Phenols (eugenol, $\beta$ -naphthol, 4-nitrophenol), acetaminophen, serotonin amines, 2-amino-5-nitro-4-fluoromethylphenol, salicylic acids	A541→G/Thr181Ala	UGT1A6*2
1A7	Phenols ( $\alpha$ -naphthol), acetaminophen, 4-methylumbelliferone, octyl gallate, propyl gallate, benzo(a)pyrene metabolites	A552→C/Thr184Ser T387→G/Asp129Lys C391→A/Arg131Lys T622→C/Tyr208Arg TATA (T57G)/none	(Ala181, Ser184) UGT1A7*1 (Asp129, Arg131, Tyr208) UGT1A7*2 (Lys129, Lys131, Tyr208) UGT1A7*3 (Lys129, Lys131, Arg208) UGT1A7*4 (Lys129, Arg131, Arg208)
1A8	Estrogens (hydroxyestrone, hydroxyestradiol, 17 $\alpha$ -ethinylestradiol), naltrexone, phenols, flavonoids, Anthraquinones, phenolphthalein, mycophenolic acid, 4-aminobiphenyl	C518→G/Ala173Gly	UGT1A8*1 (Ala173, Cys277)
1A9	Estrogens, retinoic acid, thyroid hormones, acetaminophen, SN-38 (active metabolite of irinotecan), phenols, 4-methylumbelliferone, propofol, phenols, flavonoids, anthraquinones, mycophenolic acid	G830→A/Cys277Tyr G766→A/D256N C-440→T/none T-331→C/none T-275→A/none C-2152→T/none	UGT1A8*2 (Gly173, Cys277) UGT1A8*3 (Ala173, Tyr277) None identified
1A10	Estradiol, mycophenolic acid, phenols, flavonoids	G177→A/Met59Ile C605→T/Thr202Ile G415→A/Glu139Lys C730→A/Leu244Ile	None identified
2B4	Eugenol, catechol estrogens, hyodeoxycholic acid, 1-naphthol, 4-methylumbelliferone, 5 $\beta$ -pregnane-3 $\alpha$ , 20 $\beta$ -one	T1374→A/Asp458Glu	None identified
2B7	Estrogens (4-hydroxyestrone, 4-hydroxyestradiol), androsterone, morphine, dihydromorphine, codeine, oxycodone, naloxone, naltrexone, valproic acid, serotonin, hyodeoxycholic acid, losartan	C802→T/His268Tyr	None identified
2B15	Dienestrol, phenols, flavonoids (naringenin, apigenin), anthraquinones, estrogens, (2-hydroxyestrone, 4-hydroxyestrone), bisphenol A, testosterone, dihydroxytestosterone	G253→T/Asp85Tyr	None identified
2B17	Androgens (androgen, testosterone, dihydrotestosterone, androstene-diols)	Deletion mutation	Null

Note. Adapted from Maruo et al., 2005, and Nagar and Rummel, 2006.

<sup>1</sup>Alleles have been designated in the literature based upon a single polymorphism or combination of SNPs as indicated. In some cases, no allele has been assigned other than the underlying base and amino acid changes.

more active against androgens (Belanger et al., 2003), with *UGT2B7*, *UGT2B15*, and *UGT2B17* having activity against a variety of different androgens and androgen metabolites (Chouinard et al., 2008). This activity is critical for the termination of androgen action and regulation of hormone levels in target tissues such as the prostate gland (Chouinard et al., 2008). A detailed study of the *UGT2B* subfamily involving stable transfection of each gene in a human kidney cell line devoid of hormone transferase activity found that *UGT2B7* had the broadest substrate specificity, with activity on both androgens and estrogens (Turgeon et al., 2001). *UGT2B7* had

the greatest activity towards the dihydrotestosterone (DHT) metabolite androstane-3 $\alpha$ ,17 $\beta$ -diol, whereas *UGT2B17* had the greatest activity towards androsterone, testosterone, and dihydrotestosterone.

The *UGT1* subfamily has 13 members, although only 9 of these are actually expressed. The other four contain pseudoxons that do not lead to unique proteins (Nagar and Rummel, 2006). *UGT1* gene products arise from nine different variations in exon 1; layered on top of this variability are SNPs in exon 1 that create a number of structurally distinct variants, with SNPs in upstream regions able to affect gene

transcription. Multiple SNPs can combine within a single gene to create the alleles shown in Table 3.

UGT activity is widespread across human and laboratory animal tissues, with liver generally having the greatest activity. UGTs 1A1, 1A3, 1A4, 1A6, and 1A9 are expressed primarily in liver, whereas UGTs 1A7, 1A8, and 1A10 are exclusively extrahepatic and expressed primarily in the gastrointestinal tract (Strassburg et al., 1997, 1999). A study of gene expression across 23 human tissues detected mRNAs for seven *UGT1A* enzymes and five *UGT2B* enzymes in liver (Ohno and Nakajin, 2009). Although *UGT1A1* and *UGT1A9* were the major members of the 1A subfamily expressed in liver, the levels of several *UGT2B* enzymes were considerably higher (especially *UGT2B4* and *UGT2B15*). In extrahepatic tissues *UGT1A1* was expressed in small intestine and colon (25–50% of hepatic levels), whereas the kidney has greater levels of certain enzymes (*UGT1A6* and especially *UGT1A9*) than liver. *UGT2B1* was only found in liver, whereas *UGT2B7* was the major transcript in kidney and a quantitatively important constituent in colon and small intestine. *UGT2B17* was the primary UGT in these gastrointestinal tissues. *UGT2B15* was the predominant UGT in stomach, breast, and prostate. Expression of UGT from either subfamily was very low in the lungs. These data on mRNA levels suggest a broad distribution of numerous UGTs, with *UGT2B* family members expressed to levels equal to or greater than *UGT1A* in most tissues (Ohno and Nakajin, 2009).

### 3.2. Effect of SNPs on UGT activity

A variety of studies ranging from single enzyme expression systems, liver bank studies, and *in vivo* pharmacokinetic studies have evaluated the influence of genotype on phenotype within the UGT family. Glucuronidation substrates have generally involved pharmacologic agents (morphine, lorazepam, oxazepam, valproic acid, irinotecan) and endogenous steroids depending upon the specificity of the UGT under investigation. Irinotecan has been a particular focus because this colon cancer chemotherapeutic agent has a narrow therapeutic window and relies upon glucuronidation for metabolic clearance (Lankisch et al., 2005).

Figure 3 provides an overview of functionally significant SNPs in the *UGT1A* and *UGT2B* families. SNPs that modify conjugation generally decrease activity, although certain upstream SNPs appear to be associated with modestly elevated levels of gene product.

#### 3.2.1. *UGT1A1*

This gene has three major SNPs, one in the coding region (Gly71Arg, designated *UGT1A1*\*6; rs4148323, chromosome 2:234333883), one in the conserved carboxyl-terminal domain on exon 5 (Tyr486Asp, designated *UGT1A1*\*7; rs34993780, chromosome 2:234345798), and one in an upstream regulatory sequence in which the TATA box is affected (*UGT1A1*\*28; rs8175347, chromosome 2:234333620). The coding region polymorphism is found only in Asians and is associated with decreased enzyme function, whereas the

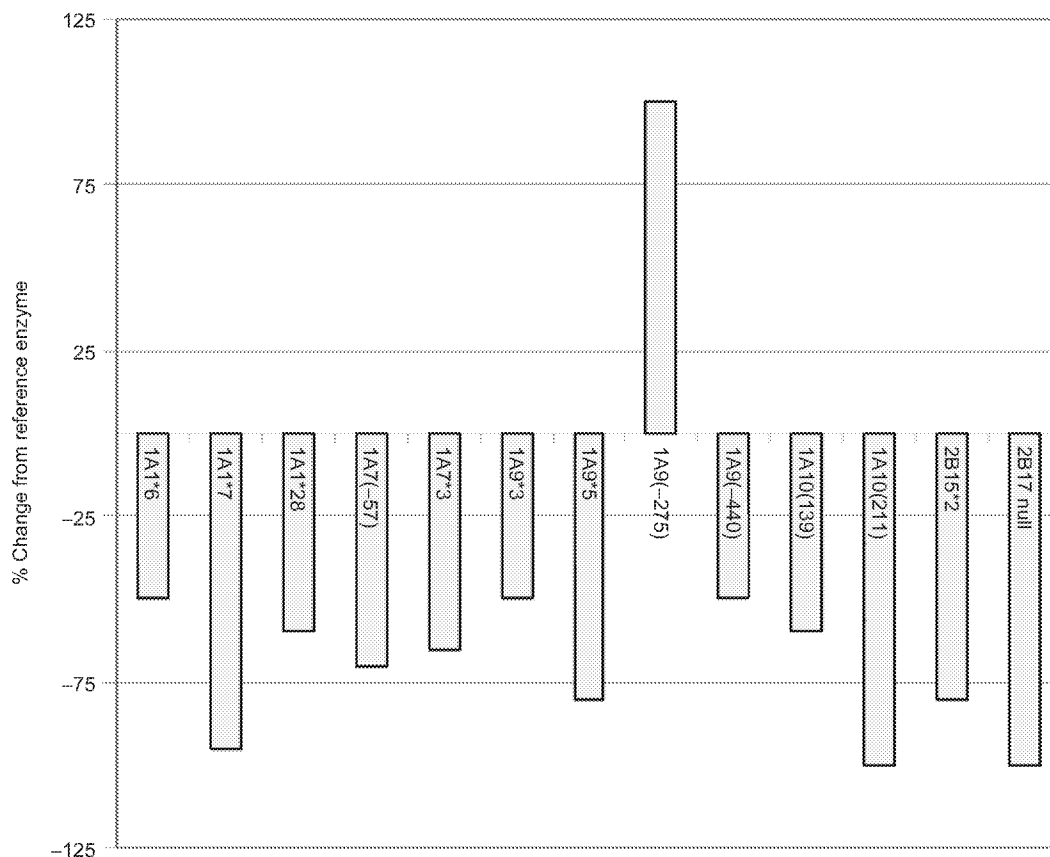


Figure 3. Influence of UGT genotype on phenotype for key variant enzymes.

promoter polymorphism is associated with decreased gene expression.

In vitro evidence in recombinant expression systems demonstrated that UGT1A1.6 was 30–70% less active than the reference enzyme in conjugating a battery of substrates (Udomuksorn et al., 2007). This is consistent with *in vivo* evidence from 37 Japanese cancer patients homozygous for the variant (*UGT1A1*\*6/\*6); this group had approximately twice the levels of circulating bilirubin as seen in individuals with the reference genotype (Sai et al., 2008). These patients also experienced increased irinotecan toxicity, with symptoms increased 2.3-fold in *UGT1A1*\*6 heterozygotes and 15-fold in homozygotes. Other studies support the importance of *UGT1A1*\*6 in modulating the *in vivo* clearance of glucuronidation substrates (Araki et al., 2006).

When the gene product containing the *UGT1A1* carboxyl-terminal SNP (*UGT1A1*\*7) was expressed in a recombinant system, the SNP was associated with a 95% loss in enzyme activity (Udomuksorn et al., 2007). This *in vitro* evidence explains why those homozygous for *UGT1A1*\*7 are more likely to experience Crigler-Najjar syndrome type II, a hyperbilirubinemia caused by impaired *UGT1A1* function. The carboxyl-terminal is the site of cofactor binding, and so this SNP may impair enzyme interaction with UDPGA (Xiong et al., 2008).

The *UGT1A1* promoter polymorphism (*UGT1A1*\*28) occurs in the TATA box such that an extra TA pair is inserted to yield a sequence of seven instead of the normal six repeat pairs (Chang et al., 2007). This thymine- and adenine-rich regulatory region is the major recognition point for the binding of transcription factors and RNA polymerase in many genes. In vitro expression systems have shown that the variant (*UGT1A1*.28) is present at 30% of the levels found for the reference gene product, suggesting a substantial effect on gene transcription (Bosma et al., 1995; Monaghan et al., 1996). A clinical condition known as Gilbert's syndrome (unconjugated nonhemolytic hyperbilirubinemia) has been attributed to the *UGT1A1*\*28 genotype in conjunction with polymorphism in *UGT1A7* (Lankisch et al., 2008b). Evaluation of glucuronidation variability using bilirubin and the SN-38 metabolite of irinotecan as substrate was conducted on

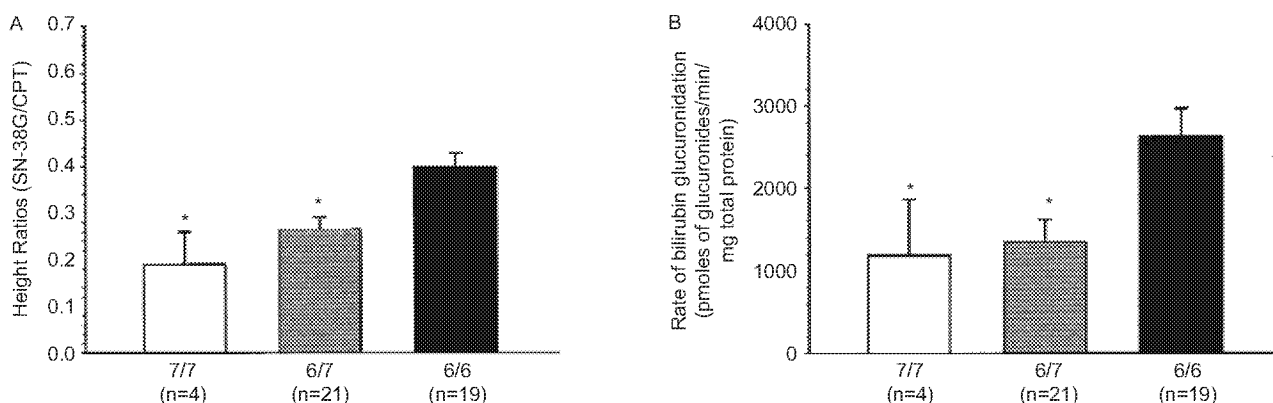
44 human liver bank microsomal samples (Iyer et al., 1999). The large degree of variability across these liver samples (2 to 3 orders of magnitude) was explained to a substantial degree by *UGT1A1*\*28. As shown in Figure 4, conjugation capacity correlated with genotype such that there was a clear gene dosage effect (Iyer et al., 1999). The decrease in mean activity in going from reference to variant gene was 2–3-fold. Greater irinotecan side effects and toxicity in these individuals was understood to involve the *UGT1A1*\*28 allele, although now other SNPs in coding and noncoding regions are being investigated as important contributors to interindividual variability in susceptibility (Iyer et al., 1999; Gagne et al., 2002; Innocenti et al., 2004; Lankisch et al., 2005).

Combination of the promoter polymorphism (*UGT1A1*\*28) and the coding region SNPs in *UGT1A1* (*UGT1A1*\*6, *UGT1A1*\*7) in the same individual may lead to low expression and low enzyme function, potentially magnifying the risk for irinotecan toxicity (Minami et al., 2007).

### 3.2.2. *UGT1A3*

Several SNPs (–66 T→C and –204 A→G) located in the promoter region that flank the TATA box have been showed to have implications for gene expression (Lankisch et al., 2008a). In a gene construct in which the SNP-modified promoter was attached to a luciferase reporter gene, the expression of the gene was decreased by approximately 25% when either the –66 or –204 SNPs were present. The implications of these SNPs on inducibility of *UGT1A3* was explored via incubation with the AH receptor agonist 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a known inducer of this UGT. TCDD produced a large induction effect in the reference gene, with this diminished only slightly (not statistically significant) when the two SNPs were present (Lankisch et al., 2008a).

Table 3 also indicates a variety of SNPs in coding regions that yield amino acid changes in *UGT1A3*. Expression of recombinant forms of these variants found little difference from the reference protein in conjugating estrone (Iwai et al., 2004). However, the combination of the W11R (rs38211242, chromosome 2:234302542) + V47A (rs6431625, chromosome 2:234302651) variants yielded UGT activity that was nearly 4-fold greater than the reference gene. Although *UGT1A3* may



**Figure 4.** Influence of *UGT1A1* promoter polymorphism (\*28) on human liver conjugation of SN-38 and bilirubin ( $N=44$ ) (reprinted from Iyer et al., 1999; with permission from Nature Publishing Group).

have some influential upstream and coding region SNPs, the implications of these SNPs on enzyme function and disease outcome need further study (Nagar and Remmel, 2006).

### 3.2.3. *UGT1A6*

*UGT1A6* is a quantitatively important enzyme that has numerous SNPs, both in the coding region and in flanking upstream regulatory sequences. Several coding region SNPs are co-expressed with the haplotype (*UGT1A6*\*2), representing the combination of the S7A (rs6759892, chromosome 2:234266408), T181A (rs2070959, chromosome 2:2234266930), and R184S (rs1105879, chromosome 2:234266941) polymorphisms along with several upstream SNPs (Krishnaswamy et al., 2005b). Expression of the *UGT1A6*\*2 coding region SNPs in a recombinant system yielded a 2-fold increase in glucuronidation capacity relative to the reference protein across a variety of *UGT1A6* substrates (serotonin, 5-hydroxytryptophol, 4-nitrophenol, acetaminophen, and valproic acid) (Krishnaswamy et al., 2005b). This is in agreement with a human liver microsome and recombinant enzyme study in which the *UGT1A6*\*2 allele was associated with greater glucuronidation activity than the reference gene product (4-nitrophenol as substrate) (Nagar et al., 2004), although Ciotti et al. (1997) found roughly a 2-fold reduction in glucuronidation activity for the *UGT1A6*\*2 allele across a number of substrates including methyl salicylate, butylated hydroxyanisole, 4-nitrophenol, a series of  $\beta$ -blockers, and methyl dopa. In a human liver bank study ( $N=54$ ) relating microsomal glucuronidation activity to *UGT1A6* genotype, only 15–20% of the interindividual variability in glucuronidation could be attributed to genotype, and in fact, there was no statistical difference between the reference and *UGT1A6*\*2 allele carriers in terms of glucuronidation activity towards serotonin (Krishnaswamy et al., 2005a). Thus, it appears that *UGT1A6*\*2 can be associated with increased, decreased, or no change in activity depending upon the test system and substrate.

### 3.2.4. *UGT1A7*

This UGT has both promoter and coding region SNPs. Similar to *UGT1A1*, the upstream TATA box contains a SNP, but instead of an extra repeat sequence, this SNP involves a T to G transversion at nucleotide position –57 (rs7586110). In studies using recombinant expression systems, this TATA box variant yielded 30% of the protein levels seen with the reference gene, suggesting that the SNP influences the ability of transcription factors to recognize or bind the promoter (Lankisch et al., 2005). This polymorphism was in linkage disequilibrium with an exon 1 polymorphism (Trp208Arg; rs11692021, chromosome 2:234255944) that is present in the two relatively common and hypomorphic alleles, *UGT1A7*\*3 and *UGT1A7*\*4 (Table 3). These variant alleles were 60–70% less active than the reference allele in recombinant expression systems probed with hydroxylated benzo(a)pyrene metabolites as glucuronidation substrate (Guillemette et al., 2000b). The TATA box variant linkage disequilibrium is possibly as high as 100%, as it has not been found in *UGT1A7*

genes that have the reference coding region. Another finding of linked inheritance is that the *UGT1A7* promoter T–57G transversion was found 97% of the time in those subjects with the *UGT1A1*\*28 promoter polymorphism (200 subjects probed) (Lankisch et al., 2005). Thus, subjects with diminished expression of *UGT1A7* can also be expected to have low expression of *UGT1A1*, a finding especially important to irinotecan toxicity because *UGT1A1* and *UGT1A7* are the major enzymes responsible for the metabolic clearance of the active metabolite (Lankisch et al., 2005).

### 3.2.5. *UGT1A8*

Thirteen coding region SNPs have been identified and grouped into haplotypes numbered \*1 to \*9 with subtypes thereof (Bernard et al., 2006). These haplotypes were tested for functional activity in a recombinant expression system against the immunosuppressive drug mycophenolate mofetil (MMF). The active metabolite of MMF, mycophenolic acid (MPA), is a glucuronidation substrate, particularly for *UGT1A8* and *UGT2B7*. Several *UGT1A8* variants were found to be less active in terms of conjugation efficiency ( $V_{\max}/K_M$ ), including the gene products from *UGT1A8*\*3 (rs17863762, chromosome 2:234191922), *UGT1A8*\*7, and *UGT1A8*\*9 haplotypes, which were constructed by site-directed mutagenesis and described in relation to the reference *UGT1A8* gene (accession number AF297093). The variant haplotypes exhibited modest declines in enzymatic efficiency of 12–24% (Bernard et al., 2006). In vivo clearance of MPA after administration of MMF failed to find an effect of the *UGT1A8*\*3 variant on MPA glucuronide formation (Levesque et al., 2007), which is not surprising given the relatively small functional change seen *in vitro*. However, the study involved only four subjects with the *UGT1A8*\*3 allele and none of these subjects were homozygotic.

### 3.2.6. *UGT1A9*

This glucuronyltransferase shares in the conjugation of a wide variety of substrates and is the predominant UGT for the intravenous anaesthetic drug propofol. A codon 766 G to A transversion in exon 1 produces an amino acid change (aspartic acid to asparagine at amino acid 256, designated *UGT1A9*\*5; rs58597806, chromosome 2:234246085) that was tested in a recombinant expression system against 3 substrates: propofol, 1-naphthol, and mycophenolic acid (Takahashi et al., 2008). This SNP decreased both the  $V_{\max}$  and  $K_M$  for propofol metabolism, with the net result being a 5-fold decrease in catalytic efficiency ( $V_{\max}/K_M$ ) relative to the reference gene product. Similar declines in catalytic function were obtained with the other glucuronidation substrates tested. When the exon 5 SNP common to numerous *UGT1A* family members (Tyr483Asp, defined as *UGT1A1*\*7 above) was tested in this system as part of *UGT1A9*, it decreased catalytic efficiency to 57% of the reference activity. This suggests that the exon 1 SNP that is unique to *UGT1A9* has a larger impact than the common exon 5 SNP (Takahashi et al., 2008). Although not tested in this system, it is plausible that the two SNPs could act in concert to yield a further lowering

of glucuronidation function. A coding region SNP (T98C) that creates the *UGT1A9*\*3 allele was evaluated in relation to the *in vivo* pharmacokinetics of the immunosuppressive drug mycophenolic acid in renal transplant patients (Kuypers et al., 2005). Although few people carried this SNP, its presence was associated with an almost 2-fold increase in parent compound area under the curve (AUC), suggesting that this SNP may also decrease enzyme activity (Kuypers et al., 2005). Glucuronidation of mycophenolic acid by *UGT1A9* is the main route for its metabolism and elimination.

Four promoter region SNPs have been identified which appear to modulate *UGT1A9* expression. Upstream SNPs at -275 (T to A) (rs6714486) and -2152 (C to T) have been shown *in vitro* and *in vivo* to lead to increased enzyme activity (Kuypers et al., 2005). Two-fold shorter drug half-life was found for mycophenolic acid in renal transplant patients carrying these upstream SNPs (Kuypers et al., 2005). Shorter drug half-life only occurred in patients taking a high dose (2 g/day), suggesting that some saturation mechanism is overcome in those who bear the promoter region SNPs and thus have greater *UGT1A9* function. Two additional upstream SNPs, C-440T (rs2741045) and T-331C (rs2741046), appear to down-regulate enzyme activity, as evidenced in a pharmacokinetic study of 40 renal transplant patients receiving mycophenolic acid (Baldelli et al., 2007). The area under the curve for this drug was 50% greater in those patients who were homozygous variant at both of these upstream loci.

### 3.2.7. *UGT1A10*

The Glu139Lys variant (rs10187694, chromosome 2:234210322) was evaluated in a recombinant expression system against three *UGT1A10* substrates: 1 naphthol, 4-nitrophenol, and 4-methylumbelliferone (Dellinger et al., 2006). The specific activity of the reference gene product (nmol/min/mg *UGT1A10* protein) was 2–3-fold greater than the variant across these three substrates. The Ile211Thr variant was found to have major consequences on enzyme function, as the recombinant variant enzyme lost all activity against a battery of glucuronidation substrates (Martineau et al., 2004).

### 3.2.8. *UGT2B4*

*UGT2B4* has a SNP in the coding region that yields an amino acid change: Asp458Glu (rs13119049, chromosome 4:70381154). This variant has been examined in several populations (Sacki et al., 2004; Levesque et al., 1999; Lampe et al., 2000), with relatively high frequency, e.g., 25%, found in Caucasians (Lampe et al., 2000). However, there has been limited characterization of the SNP effect on enzyme function (Levesque et al., 1999) with data comparing activity between reference and variant genes lacking. Another variant allele, *UGT2B4*\*3 (accession number AF081793), was initially characterized as a new UGT enzyme, *UGT2B11* (Jin et al., 1993), but later recognized as a variant of *UGT2B4*. It contains two coding region SNPs leading to amino acid changes at positions 109 and 369 (both phenylalanine to leucine). When tested in a recombinant expression system, it was found to have broad

glucuronidation capacity except against hydrodeoxycholic acid (Jin et al., 1993) and so may have somewhat diminished activity relative to the reference *UGT2B4* gene product. However, this has not been evaluated.

### 3.2.9. *UGT2B7*

A coding region amino acid change involving codon 802 (T to C) yields the Tyr268His variant designated as *UGT2B7*\*2a (rs7439366, chromosome 4:69998927). This variant has been studied in association with enzyme activity and adverse health outcomes in several cancer epidemiology studies. The evidence that this amino acid change has an effect on enzyme activity is mixed. A study of 86 patients taking morphine showed greater glucuronidation activity (higher ratio of metabolite to parent) in those with the variant genotype (Sawyer et al., 2003). However, a similar study of 70 cancer patients taking morphine for pain failed to find a difference in morphine glucuronidation across *UGT2B7* genotypes (Holthe et al., 2002). *UGT2B7* is one of the UGTs involved in tamoxifen metabolite conjugation, with evidence for the *UGT2B7.2a* variant having 2–5-fold less activity for conjugating various metabolites of tamoxifen in expression systems when compared to the reference gene (Blevins-Primeau et al., 2009). Further, an influence of *UGT2B7* polymorphism on tamoxifen metabolism was suggested by analyzing 111 human liver bank samples wherein the glucuronidation of tamoxifen metabolites was decreased 27% in samples from donors who were homozygous variant (Blevins-Primeau et al., 2009). However, another liver bank study involving samples from 91 Caucasian subjects failed to detect a difference in the microsomal conjugation of several different UGT substrates (androsterone, menthol, morphine) in relation to the *UGT2B7*\*2a allele (Bhasker et al., 2000). Other *in vivo* evidence with an androgen metabolite (androstane-3,17-diol) found that glucuronidation of this metabolite was slightly increased in the subjects homozygous for the variant gene (Swanson et al., 2007).

The mixed nature of the *UGT2B7*\*2a influence on enzyme function may be related to its linkage to an upstream SNP, -139G→A (rs73823859) designated as *UGT2B7*\*2g. This regulatory sequence SNP resulted in substantial declines in gene expression in two recombinant systems (Duguay et al., 2004a). The presence of this SNP was probed in 175 patients on long-term morphine therapy. Six patients were identified as heterozygotes for this SNP; these patients had significantly lower morphine glucuronidation ratios in serum than the 169 reference genotype individuals. Although the frequency and functional implications of this SNP need further evaluation, its linkage to the coding region SNP (*UGT2B7*\*2a) may be an additional influence on phenotype not accounted for in the studies of the coding region SNP.

Another 5' upstream polymorphism in *UGT2B7* occurs at -900 (G to A) (rs7438135). When a pharmacokinetic study was conducted in 20 sickle cell patients administered morphine, morphine glucuronidation was influenced by this upstream SNP (Darbari et al., 2008). Homozygous variant subjects (A/A) exhibited 50% higher blood concentrations of

conjugate relative to parent compound than in the combined reference plus heterozygote group. This suggested enhancement in gene expression in association with the upstream SNP merits further evaluation.

### 3.2.10. *UGT2B15*

This enzyme has a coding region SNP involving an amino acid change at position 85 (Asp to Tyr; rs1902023, chromosome 4:69570689). Expression of the reference gene (*UGT2B15.1*) and the variant (*UGT2B15.2*) in a human kidney cell line revealed similar  $K_M$  values for both enzymes against dihydroxytestosterone and androstane-3 $\alpha$ ,17 $\beta$ -diol (Levesque et al., 1997). However, the variant was associated with somewhat greater  $V_{max}$ . A study evaluating androgen balance *in vivo* found that serum levels of androstanediol-17-glucuronide appeared to be dependent upon this polymorphism with reference individuals (Asp/Asp) having approximately 50% higher conjugate levels than the variant (Tyr/Tyr) (Swanson et al., 2007). This result suggests lower activity in the variant case, opposite of that expected based upon the earlier *in vitro* result with this substrate (Levesque et al., 1997). Other results with this SNP tend to favor the interpretation that the variant *UGT2B15\*2* form is associated with less activity. The (S)-isomer of oxazepam is specifically conjugated by *UGT2B15* whereas the (R)-isomer has less UGT specificity. In an *in vitro* expression system (S)-oxazepam glucuronidation was 5-fold lower with the variant enzyme (Court et al., 2002). Further, (S)-oxazepam conjugation was correlated with *UGT2B15* genotype in a study of 54 human liver bank samples (Court et al., 2004). Livers from donors homozygous for the *UGT2B15\*2* allele were 2.7-fold lower in (S)-oxazepam conjugation with heterozygotes intermediate. A gender-based difference was also observed with activity significantly greater in males (1.7-fold). An *in vivo* study of lorazepam pharmacokinetics in 24 healthy volunteers found variant *UGT2B15\*2/\*2* individuals to have 40–50% slower systemic clearance than individuals with the reference genotype (Chung et al., 2005). Overall, the *UGT2B15\*2* allele appears to decrease activity by up to 5-fold *in vitro* with an effect also demonstrable *in vivo* (Swanson et al., 2007; Chung et al., 2005).

### 3.2.11. *UGT2B17*

This enzyme has a deletion polymorphism in which much of the coding region is missing from the gene. This is an inactivating change such that homozygotes lack this activity. This effect was seen in a hormone evaluation of 615 volunteers in which the balance between androgens and their glucuronide metabolites, both in blood and urine, was evaluated (Swanson et al., 2007). Of the circulating androgens, only one androgen metabolite, androstane-3 $\alpha$ ,17 $\beta$ -diol, was influenced by the deletion polymorphism. Individuals with the reference genotype had, on average, 50% greater serum content of the 17 $\beta$ -glucuronide, which is consistent with the expectation that the variant will lack glucuronide conjugation. Regarding excreted androgens, a major finding was that urinary testosterone was 15-fold lower in deletion polymorphism homozygotes. Because the assay of urinary testosterone involves  $\beta$ -glucuronidase treatment, it is a measure of total testosterone in urine. Further, because less than 1% of excreted testosterone is unconjugated, this study shows that urinary excretion of testosterone is largely dependent upon *UGT2B17*-mediated conjugation and this can be prevented by the deletion polymorphism. There was also an effect on DHT excretion, but this was much smaller and not statistically significant (Swanson et al., 2007). This polymorphism has also been shown to influence the fate of the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone by modifying the glucuronidation of its alcohol metabolite, the 1-butanol, NNAL (Gallagher et al., 2007b). This effect and the implication for lung cancer risk are further discussed below.

### 3.3. Frequency of enzyme variants

SNPs in UGTs are common, with Figure 5 highlighting the frequency of key SNPs that have been shown to impact enzyme function or levels of the gene product. The most extensively studied *UGT1A1* polymorphism is the TATA box sequence insertion, which has a relatively low allele frequency in Asians, but frequencies in the 40% range in African Americans and Caucasians (Kaniwa et al., 2005). In contrast, the *UGT1A1\*6* allele is not found in Caucasians or African Americans but is common in Asian populations (15.7% in

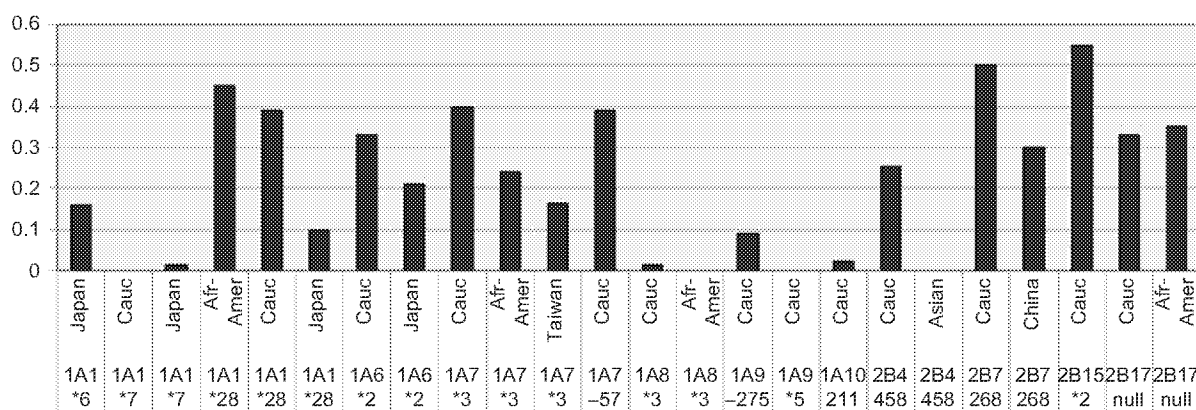


Figure 5. Allele frequency for UGT polymorphisms. Allele number or SNP codon location shown on X axis along with ethnicity.

Japanese). The *UGT1A1*\*7 allele, which involves a SNP in the carboxyl terminal region, has not been widely characterized. A study in a healthy Japanese population found it in only 2 of 71 subjects (Takeuchi et al., 2004a). Further, genotyping of 65 Caucasian gastrointestinal tract tumor patients failed to find any subjects with the *UGT1A1*\*7 allele.

Figure 5 also points out that *UGT1A6*\*2 and *UGT1A7*\*3, as well as the upstream regulatory SNP in *UGT1A7* (−57 position) are all commonly seen. The *UGT1A7*\*4 variant as well as *UGT1A8* variants (\*3, \*7, \*9) and *UGT1A9* coding region SNPs (\*3, \*5) all have substantial functional impact but have very low frequency in Caucasians and African Americans (1% or less) (Butler et al., 2005; Bernard et al., 2006; Levesque et al., 2007; Paoluzzi et al., 2004). The *UGT1A9* upstream SNP conferring increased gene expression, −275 (T to A), has been found in Caucasians at an allele frequency of 9% but other SNPs in *UGT1A9* and *UGT1A10* were found very infrequently (<1%) in Asians and Caucasians (Thomas et al., 2006). Figure 5 shows representative results for a few of these low-frequency alleles.

The *UGT2B* subfamily has SNPs in high frequency for *UGT2B4* (Caucasians but not in Asians), *UGT2B7*, *UGT2B15*, and *UGT2B17*. Regarding the *UGT2B15*\*2 allele, it is commonly found in Caucasians as indicated by several reports (Levesque et al., 1997; Park et al., 2004; Swanson et al., 2007; Court et al., 2004), with data from African Americans and Asians also indicative of high frequency (Court et al., 2004). The *UGT2B17* deletion polymorphism is commonly found in Caucasians and African Americans such that the homozygous variant, double-null genotype can be expected to occur in 10% of the population.

### 3.4. Additional sources of variability in UGT activity

UGT expression and activity are modulated by a number of endogenous and exogenous factors. Many compounds that can induce oxidative biotransformation enzymes can also induce UGTs. This was studied in detail with 18 different inducers of CYPs that act through at least five different activation pathways responsible for inducing *CYP1A1* (aryl hydrocarbon receptor ligands, e.g., 2,3,7,8-TCDD), *CYP2B* (constitutive androstane receptor [CAR] ligands, e.g., phenobarbital, nonplanar polychlorinated biphenyl [PCBs]), *CYP3A* (pregnane X receptor [PXR] ligands, e.g., glucocorticoids), *CYP4A* (peroxisome proliferator-activated receptor  $\alpha$  ligands, e.g., clofibrate, diethylhexylphthalate), and *CYP2E* (no specific transcription factors identified—inducers tested are streptozotocin, isoniazid) (Shelby and Klaassen, 2006). Also evaluated were agents that activate antioxidant defense systems and conjugation enzymes without activating CYPs (olitpraz, ethoxyquin). Administration of these various agents to rats generally increased UGT mRNA levels in liver but not in duodenum, with the CAR and PXR ligands the most active. Each UGT enzyme had its own activation profile such that hepatic *UGT1A1* was induced most by ethoxyquin, olitpraz, and acetyl salicylic acid, whereas *UGT2B1* was most induced by *trans*-stilbene oxide and PCB congener 99. Thus although many CYP inducers also activate UGTs, the pattern found in

this rat study is complex and varies according to UGT enzyme and tissue (Shelby and Klaassen, 2006). Evaluation of human UGT induction came from a study of transgenic mice containing the full complement of human *UGT1A* genes (Chen et al., 2005). Various UGTs were induced from the administration of the AH receptor ligand 2,3,7,8-TCDD, and the PXR activators dexamethazone and pregnenolone-16-carbonitrile (PCN).

When *UGT1A4* inducibility by AH receptor ligands was evaluated in HEPG2 cells, those cells containing the *UGT1A4* reference gene showed 4.4-fold induction when incubated with 3-methylcholanthrene and 10.4-fold with 2,3,7,8-TCDD (Erichsen et al., 2008). However, the induction of *UGT1A4* was ablated by upstream variants, which suggests gene-environment interaction in the regulation of expression of this UGT.

Glucuronidation capacity can also be affected by lifestyle and dietary factors as illustrated for alcohol and cruciferous vegetables. A liver bank study evaluating genotype and phenotype across 54 samples found that *UGT1A6* activity was modulated by history of alcohol consumption (Krishnaswamy et al., 2005a). Subjects who drank more than 14 drinks per week had 2 times greater serotonin conjugating activity as well as greater liver *UGT1A6* protein and mRNA content.

Cruciferous vegetables are known inducers of a variety of conjugation enzymes with evidence that *UGT1A1* is involved in this inductive response. A 2-week trial of a high cruciferous vegetable diet in 70 volunteers found associated decreases in serum bilirubin (Navarro et al., 2009). However, this occurred to a similar extent in those with reference and variant (*UGT1A1*\*28) genotype, suggesting that the cruciferous vegetable effect may not be entirely through *UGT1A1* or that the *UGT1A1*\*28 SNP (which affects gene regulation) does not affect this response (Navarro et al., 2009).

A variety of drugs can participate in drug-drug interaction at the level of competitive or noncompetitive inhibition of glucuronidation, including probenecid (noncompetitive inhibition), tranilast (antiasthma medication associated with hyperbilirubinemia due to inhibition of *UGT1A1*), and sulfinpyrazone, which can inhibit a number of UGT enzymes (Riches et al., 2009).

Another source of variability is the ontogeny of UGTs, as they are immature at birth and develop over the first several months of life (Alcorn and McNamara, 2002). This delayed development of UGT expression leads to greater risk for hyperbilirubinemia in neonates along with a greater susceptibility to toxicity from chloramphenicol, which is dependent upon glucuronidation for clearance (Mulhall et al., 1983; Vest, 1965). Pediatric pharmacokinetic trials of six drugs cleared by glucuronidation without prior oxidative metabolism found decreased drug clearance in neonates, with this rising to adult levels by 6 months of age (Ginsberg et al., 2002b). Sulfotransferases have more rapid ontogeny than glucuronidases and are active at birth. This can lead to a preponderance of sulfate conjugation in early life that switches to glucuronidation later in childhood. This pattern has been demonstrated for acetaminophen and is plausible

for other UGT substrates (Levy et al., 1975; Miller et al., 1976; Allegaert et al., 2005).

### 3.5. UGT Modulation of endogenous hormones and xenobiotics

As described above, UGTs are key conjugation enzymes for the glucuronidation of numerous endogenous substrates, including a variety of steroids, estrogens, catechol estrogen metabolites, androgens, and their metabolites, bile acids, and bilirubin (Maruo et al., 2005). Thyroid hormones are also substrates for glucuronidation reactions, with the phenolic and carboxylic functional groups on thyroxine and triiodothyronine being points of conjugation (Tong et al., 2007). UGT expression systems found that both the phenolic and carboxylic conjugation reactions were most efficient for *UGT1A3*, *UGT1A8*, and *UGT1A10*, with some activity also from *UGT1A1*.

Glucuronidation of these endogenous compounds generally leads to deactivation and removal because they are rendered more water soluble. Sulfation is at times a competing conjugation pathway that can allow storage and recirculation of endogenous hormones (Nakamura et al., 2005). In contrast, glucuronidation is generally a direct deactivation and removal step, although it is possible that  $\beta$ -glucuronidases may reactivate conjugates in the intestines for chemicals undergoing enterohepatic recirculation, and systemically in tissues rich in  $\beta$ -glucuronidase (e.g., placenta) (Lucier et al., 1977).

#### 3.5.1. Conjugation of pharmacological agents and toxicants

As described above, many therapeutic drugs are substrates, either directly or as an oxidized metabolite, for glucuronidation. In some cases, drug glucuronidation has specificity for a particular enzyme and so it can be used as a probe of the function of that enzyme relative to polymorphisms (e.g., oxazepam/lorazepam and *UGT2B15*: Chung et al., 2005; irinotecan and *UGT1A1*: Iyer et al., 1999; mycophenolic acid and *UGT1A9*: Kuypers et al., 2005; morphine and tamoxifen for *UGT2B7*: Sawyer et al., 2003; Darbari et al., 2008; Blevins-Primeau et al., 2009). However, in many cases the drug can be glucuronidated by numerous UGTs (e.g., acetylsalicylic acid: Kuehl et al., 2006), limiting its utility as a probe drug for specific enzymes. Even in those cases where there is specificity it is not absolute and so the evaluation of genotype effect on phenotype will be affected by the presence of other enzymes in the system. For this reason most genotype-phenotype studies utilize recombinant expression systems so that a single enzyme (and its polymorphisms) can be studied in isolation. Additionally, overlapping substrate specificity can mute the effects of a particular polymorphism on *in vivo* drug pharmacokinetics as exemplified by *UGT1A6*\*2 and deferiprone, in which there was no effect on pharmacokinetics in spite of *in vitro* evidence of the importance of genotype (Liment et al., 2008). This issue underlies the fact that relatively few examples exist where UGT polymorphism clearly affects *in vivo* drug clearance. Perhaps the best example is in the case

of irinotecan for which the package insert now includes a warning concerning reduced clearance in the case of *UGT1A1* polymorphism (Perera et al., 2008).

Many environmental toxicants are detoxified and excreted via glucuronide conjugation, either as the parent compound or oxidized metabolite. In some cases, the specific UGTs responsible for this metabolism have been explored. For example, the endocrine active plasticizer bisphenol A (BPA) is excreted primarily as the glucuronide metabolite in humans and rodents (Dekant and Volkel, 2008). Evaluation of the rat liver UGTs responsible for BPA glucuronidation via immunoinhibition studies revealed that 65% of this activity was associated with *UGT2B1* (Yokota et al., 1999). This finding was supported by data showing that hormonal regulation of hepatic BPA conjugation in rats (down-regulated by androgens) is associated with decreased *UGT2B1* expression (Takeuchi et al., 2004b). In humans *UGT2B15* appears to be the predominant enzyme for BPA conjugation, as it had the greatest catalytic activity at both low and high substrate concentrations (Hanoika et al., 2008).

The carcinogenic heterocyclic amine produced in well-done cooked meat, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), and its hydroxylated metabolite (*N*-hydroxy-PhIP), are metabolized principally by *UGT1A* enzymes (Malfatti et al., 2005; Malfatti and Felton, 2004). In recombinant expression systems involving the eight functional human *UGT1A* enzymes, all enzymes except for *UGT1A6* exhibited conjugation activity with *N*-hydroxy-PhIP as substrate (Malfatti and Felton, 2004). *UGT1A1* was clearly the most active, being 286 times more active than the enzyme with the lowest detectable activity (*UGT1A7*), and 5 times more active than *UGT1A4*, the second most active enzyme. The implications for toxic mechanism of action have been explored in that Gunn rats deficient in *UGT1A* enzymes exhibited lower levels of glucuronidated metabolites and higher levels of hepatic DNA adducts than Wistar rats (*UGT1A* proficient) when given a 100  $\mu$ g/kg oral dose of [<sup>14</sup>C]PhIP (Malfatti et al., 2005). Interestingly, the colon DNA adduct response was the opposite, suggesting that deficient hepatic glucuronidation decreases the amount of metabolite reaching the gastrointestinal tract where  $\beta$ -glucuronidases would normally regenerate the active mutagen.

Incubation of benzidine or its *N*-acetylated metabolite (*N*-acetylbenzidine) with recombinant UGTs demonstrated a rank order of activity as follows: *UGT1A9* > *UGT1A4* >> *UGT2B7* > *UGT1A6* ~ *UGT1A1* (Zenser et al., 2002). Although *UGT2B7* was not the most active UGT against benzidine *in vitro*, molecular epidemiology studies in bladder cancer cases involving benzidine exposure have focused upon polymorphisms in this enzyme (Lin et al., 2005; Zimmerman et al., 2008).

Another toxicant relying upon glucuronidation for detoxification and removal is the tobacco-specific nitrosamine metabolite NNAL. As described above, *UGT2B17* appears to be important *in vivo*, as evidence from women smokers shows that those with the deletion polymorphism have a lowered glucuronide to NNAL ratio in urine (Gallagher et al., 2007b).

Extending the study to lung cancer risk, women homozygous for the deletion polymorphism ( $N=58$ ) had a doubling in their incidence of lung cancer, which was in contrast to males who saw no change in lung cancer risk in relation to the polymorphism ( $N=60$ ) (Gallagher et al., 2007b). However, other UGTs also appear to be involved, with evidence supporting roles for *UGT1A4*, *UGT1A9*, *UGT2B7*, and *UGT2B10* (Wiener et al., 2004a, 2004b; Chen et al., 2008).

Oxidized metabolites of polycyclic aromatic hydrocarbons, some of which are important species in metabolic activation pathways, have also been shown to be substrates for particular UGTs. For example, glucuronidation of benzo(a)pyrene *trans*-7,8-diol, the precursor to the carcinogenic 7,8-diol-9,10-epoxide, was studied in a recombinant expression system in which multiple UGTs showed activity (Fang et al., 2002). *UGT1A10* showed the lowest  $K_M$ , followed by *UGT1A9*, *UGT1A1*, *UGT2B7*, and *UGT1A7*, although this varied somewhat depending upon exactly which glucuronide diastereomer was formed. The importance of *UGT1A10* in benzo(a)pyrene conjugation was further demonstrated with recombinant enzymes screened against the 3-hydroxy, 7-hydroxy, and 9-hydroxy metabolites of benzo(a)pyrene (Dellinger et al., 2006). Each of these oxidized polyaromatic hydrocarbon (PAH) metabolites were preferentially glucuronidated by *UGT1A10*, as demonstrated by the greater  $V_{max}/K_M$  ratio for this enzyme compared to the others tested. *UGT1A10* is an extrahepatic enzyme found extensively in aerodigestive tract tissues (e.g., mouth, larynx, esophagus) (Zheng et al., 2002). Regarding hepatic metabolism of oxidized benzo(a)pyrene metabolites, *UGT1A1* and *UGT1A9* appear to be most active. In vitro studies with human liver microsomes show that individuals with the *UGT1A1*\*28 (TATA box) variant are deficient in *UGT1A1* microsomal protein content, bilirubin conjugation, and in the glucuronidation of benzo(a)pyrene 7,8-diol (Fang and Lazarus, 2004).

### 3.6. Epidemiological associations

The common occurrence of UGT polymorphisms and their ability to modulate conjugation capacity has led to their evaluation in molecular epidemiology studies in relation to risk for several different types of cancer and other health effects. The established role of the *UGT1A1* promoter polymorphism (*UGT1A1*\*28) in Gilbert's syndrome (unconjugated hyperbilirubinemia) and the suggested association of this SNP with gallbladder disease in children (Lankisch et al., 2008b; Carpenter et al., 2008) indicate the importance of *UGT1A1* in conjugating bilirubin. Further, this evidence suggests that SNPs in *UGT1A1* can have a clinically important effect on glucuronidation capacity. However, the evidence for other health endpoints is not as definitive.

#### 3.6.1. Breast and endometrial cancers

The finding that *UGT1A1* as well as other UGTs conjugate estradiol (Guillemette et al., 2000a) has led to the hypothesis that SNPs causing low UGT function will increase circulating levels of estrogens and increase breast cancer risk (Nagar and Rummel, 2006). Several studies support this hypothesis; for

example, a study of 200 African American women found a 1.8-fold elevation in breast cancer risk in those carrying the low-activity *UGT1A1*\*28 genotype (Guillemette et al., 2000a). A larger Chinese study similarly found a 1.7-fold increased breast cancer risk in those carrying the *UGT1A1*\*28 allele, although this association only existed in cases occurring under the age of 40 (Adegoke et al., 2004). However, the results have been mixed as one study involving 455 Caucasian breast cancer cases found no association with *UGT1A1* genotype (Guillemette et al., 2001), whereas another study of 163 breast cancer cases found a decreased risk in subjects with the *UGT1A1*\*28 allele (Sparks et al., 2004). Thus, although the *UGT1A1* genotype can be a susceptibility factor in breast cancer, the multifactorial nature of this disease may lead to other factors being more influential in certain cases (Nagar and Rummel, 2006).

*UGT2B4* was evaluated with respect to polymorphism and breast cancer risk. Although it is primarily expressed in the liver, *UGT2B4* polymorphism at the 458 position (Asp to Glu) was evaluated in breast cancer patients and controls (Sparks et al., 2004). The relevance to breast cancer stems from the fact that *UGT2B4* is capable of conjugating potentially carcinogenic estrogen metabolites (catechol estrogens). However, in a study of 163 patients there were no associations between cancer incidence and *UGT2B4* genotype (Sparks et al., 2004). As stated above, the implications of this SNP on UGT function needs clarification.

A suggestive link between the *UGT1A1*\*28 allele and endometrial cancer was found in 222 women with this disease (Duguay et al., 2004b). The *UGT1A1*\*28 allele was associated with a decreased cancer rate, with this being attributed to less glucuronidation of 2-hydroxyestradiol, a known antiproliferative endogenous factor in the endometrium (Duguay et al., 2004b; Nagar and Rummel, 2006).

#### 3.6.2. Bladder cancer

Several studies have examined the association between *UGT2B7*\*2 and bladder cancer in cases with likely exposure to benzidine. A study of 36 bladder cancer cases in Chinese dye industry workers found the homozygous variant genotype (268 Tyr/Tyr) to be 3.3-fold more likely in cancer cases than in controls (Lin et al., 2005). However, a study attempting to replicate this finding in 171 Caucasian bladder cancer cases in workers with a history of aromatic amine exposure failed to find a relationship between the 268Tyr/Tyr genotype and cancer risk (Zimmerman et al., 2008). This difference in results may be related to ethnicity (Chinese versus German Caucasians), differences in degree of historic aromatic amine exposure, and a variable role for *UGT2B7* in relation to other UGTs in conjugating the amines (*UGT2B7* is a relatively minor UGT in benzidine metabolism *in vitro*: Ciotti et al., 1999). Further, because *UGT2B7* conjugation forms acid labile *N*-glucuronide metabolites of benzidine, this can be seen as an activating step for bladder carcinogenesis because more metabolite would be targeted for aromatic amine regeneration in the bladder (Ciotti et al., 1999). Thus, although the Chinese data are suggestive of a risk-enhancing effect for

the *UGT2B7* polymorphism, this was not supported in the German occupational study and it does not necessarily fit with the mechanism of action for aromatic amine-induced bladder cancer.

### 3.6.3. Upper GI tract

*UGT1A7* is most heavily expressed in upper gastrointestinal (GI) tract tissues such as esophagus and orolaryngeal tissues (Ohno and Nakajin, 2009), leading to the suggestion that these tissues would be more vulnerable to inhaled or ingested toxicants if the low-activity forms of the enzyme were present. This hypothesis is supported by evidence from 194 orolaryngeal cancer cases and 388 controls in which the reference genotype was statistically more prevalent in the controls and the low-activity *UGT1A7*\*2, *UGT1A7*\*3 and *UGT1A7*\*4 variants were more likely in cases (odds ratios 3.7–6.2) (Zheng et al., 2001). This was only true in smokers. Another upper gastrointestinal tract study of 76 cancer cases and 210 controls was consistent in showing the *UGT1A7*\*3 allele with an elevated risk (odds ratio = 2.02,  $p < .001$ ) (Vogel et al., 2002) (article in German, English abstract only).

### 3.6.4. Colorectal cancer

*UGT1A6* polymorphism has been studied with respect to colon adenomas by several investigators (Bigler et al., 2001; Chan et al., 2005). Aspirin inhibition of the prostaglandin pathway involving cyclooxygenase (COX-2) is a potential benefit for colorectal cancer because of the overexpression of this enzyme in colon cancer cells (Chan et al., 2007). Because *UGT1A6* can conjugate and clear aspirin, these studies have focused on the *UGT1A6* genotype among patients who were taking aspirin. Both Bigler et al. 2001 (474 colon adenoma cases) and Chan et al. 2005 (1062 colon adenoma cases) found decreased risk of colon cancer progression or recurrence in those taking aspirin and carrying the *UGT1A6*\*2 allele. *UGT1A6*\*2 appears to diminish salicylate conjugation (Ciotti et al., 1997). This suggests that lower rates of aspirin conjugation will increase its circulating levels and efficacy in preventing colorectal cancer.

Low-activity *UGT1A7* variants, particularly *UGT1A7*\*3, have been associated with increased risk for colorectal cancer across several studies (Butler et al., 2005; Tang et al., 2005; Strassburg et al., 2002). One study evaluated *UGT1A7* genotype and heterocyclic amine dietary ingestion in relation to colon cancer and found an increasing risk with low-activity genotype (*UGT1A7*\*3) and high amine ingestion (Butler et al., 2005). Two other studies just looking at genotype and colorectal cancer without exposure assessment also found the *UGT1A7*\*3 allele associated with this endpoint: an odds ratio of 2.75 in 78 Caucasian cases (Strassburg et al., 2002), and in 268 cases, an odds ratio of 2 for colorectal cancer and of 4.9 for metastases of colorectal cancer in those carrying the *UGT1A7*\*3 allele (Tang et al., 2005).

### 3.6.5. Hepatocellular carcinoma (HCC)

*UGT1A7* polymorphism has been associated with increased risk for HCC in three studies. The first involved 59 cases and

70 controls in which low-activity variants were present in the vast majority of cases (93%), leading to an odds ratio of 10.8 (Vogel et al., 2001). A study of 217 HCC cases and 291 controls in Taiwan found a strong effect of hepatitis B or C infection, with *UGT1A7* polymorphism also an independent risk factor for HCC development (odds ratio for homozygote *UGT1A7*\*2 or *UGT1A7*\*3 = 3.06); *UGT1A7* polymorphism was also associated with earlier age of HCC onset (50 years old in variants, 59 in reference individuals) (Tseng et al., 2005). The third study was conducted in 280 Japanese hepatitis C-infected patients, 120 of whom had HCC (Wang et al., 2004). The occurrence of HCC was associated with low-activity *UGT1A7* alleles (homozygote odd ratio = 2.73; heterozygote odds ratio = 1.80). With respect to viral hepatitis and hepatic cirrhosis, Tang et al. (2008) studied 159 Taiwanese cirrhosis patients. Infection with hepatitis B or C virus significantly contributed to cirrhosis risk, along with an independent contribution from low-activity *UGT1A7* polymorphisms. Given that *UGT1A7* is able to conjugate tobacco-related nitrosamines and PAH carcinogenic metabolites, it may be an important detoxification mechanism for a variety of agents that can cause toxicity and cancer in the liver.

### 3.6.6. Prostate cancer

*UGT2B17* has been investigated as a potential modulator of prostate cancer risk, a hypothesis stemming from this enzyme's ability to conjugate a range of androgens and because of its localization to this tissue (Park et al., 2006). Theoretically, subjects carrying the low-activity allele might have greater free androgen and increased cancer risk. This has been suggested in one study in which the deletion polymorphism was statistically associated with increased prostate cancer in Caucasians (odds ratio 1.9, 95th range 1.2–3.0) but not in African Americans. However, a study of 411 Caucasian prostate cancer cases failed to find an association of the disease and the *UGT2B17* deletion polymorphism (Gallagher et al., 2007a).

## 3.7. Summary: Risk implications of UGT polymorphism and potential for distributional analysis

### 3.7.1. Is the enzyme important to toxicant action?

This can vary across xenobiotics depending upon the rate of different competing pathways for chemical disposition and, within conjugation systems, the degree of overlapping substrate specificity with other conjugating enzymes. In particular, UGTs may overlap with sulfotransferases, which may compensate for diminished UGT activity due to early life immaturity or inactivating polymorphisms. Although there are examples of diminished *in vivo* clearance of pharmacologic agents due to UGT polymorphism (e.g., *UGT1A1* polymorphism and irinotecan metabolite clearance), this will not always occur due to alternative clearance pathways (e.g., SULTs, other UGT enzymes) and so the implications of UGT polymorphism for internal dose need to be evaluated on a case-by-case basis.

The cases in which UGT polymorphism may have the greatest implications for human health risk are (1) for substrates

that have no alternative enzyme systems to compensate for deficiency in a particular UGT; (2) under conditions of high-dose exposure wherein the capacity of alternative (e.g., SULT) pathways are exceeded; (3) where there is simultaneous exposure to multiple xenobiotics leading to an interaction that decreases conjugation function (competitive or noncompetitive inhibition, depletion of conjugation cofactors), making the system more vulnerable to a further decrease in function from genetic polymorphism. Although this type of interaction has been demonstrated between drug glucuronidation substrates (e.g., acetaminophen-probenecid-sulfinpyrazone; lorazepam-valproic acid) (Riches et al., 2009; Chung et al., 2008), it has not been explored for drug-toxicant interaction within the context of UGT polymorphism.

Molecular epidemiology evidence suggests that specific UGT polymorphisms can cause hyperbilirubinemia syndromes and may modulate risk in certain types of cancer.

### 3.7.2. Are there influential SNPs of sufficient frequency to have a broad impact?

As depicted in Figure 3, there are a variety of SNPs in different UGTs that can influence enzyme activity. Most influential SNPs have produced a decrease in UGT activity either via a partial decrease in function (e.g., *UGT1A7\*3*), gene deletion (*UGT2B17 null*), or via SNPs in upstream regulatory sequences (e.g., *UGT1A1\*28*). The most heavily studied SNP is *UGT1A1\*28* because the *UGT1A1* enzyme is essential to the hepatic conjugation of bilirubin and numerous other substrates, with extrahepatic expression as well. Further, the *UGT1A1\*28* allele is relatively common across ethnic groups (Figure 5) and has shown diminished activity in expression systems, diminished glucuronidation capacity in liver bank studies and increased susceptibility to irinotecan toxicity in patients taking this medication.

Additional SNPs of potential importance to risk assessment are presented in Table 4. These have been prioritized based upon their influence on UGT function and their frequency of occurrence. This table integrates the information presented in Figures 3 and 5 along with evidence for genotype influence on *in vivo* pharmacokinetics or disease outcome. The table highlights three *UGT1A1* alleles (*UGT1A1\*6*, *UGT1A1\*7*,

*UGT1A1\*28*), the *UGT1A7\*3* allele, and two *UGT2B* alleles (*UGT2B15\*2*, *UGT2B17 null*). Other UGTs had more limited or conflicting evidence pertaining to genotype effect on phenotype (*UGT1A3*, *UGT1A6*, *UGT2B4*, and *UGT2B7*). Several UGTs that appear to influence enzyme function based upon *in vitro* evidence have too low a frequency (<1%) for listing in Table 4 (*UGT1A7\*4*, *UGT1A8* SNPs, *UGT1A9\*3*, *UGT1A9\*5*, and *UGT1A10* SNPs).

### 3.7.3. Can the polymorphism data inform health risk assessments?

For *UGT1A1\*28* and *UGT2B15\*2*, data are available to not only characterize genotype effect on phenotype in a highly relevant system (microsomes from human liver bank samples), but also indicate within genotype variability in enzyme activity (Iyer et al., 1999; Court et al., 2004). For other influential UGT SNPs presented in Table 4, the data come primarily from *in vitro* expression systems, which compare activity between variants and reference genotype but do not indicate heterogeneity across individuals. However, it's possible that risk assessments may use the recombinant enzyme data along with allele frequency to make simple adjustments of pharmacokinetic models to screen for the influence of genotype on internal dose and risk.

### 3.7.4. Outstanding questions regarding the utility of UGT polymorphism data for human risk assessment

Although all of the UGT polymorphisms in Table 4 are candidates for incorporation into risk assessments, only those known to conjugate the specific toxicant of interest or its metabolite would be relevant for a given assessment. Thus far there are relatively few examples where toxicants have been tested against a battery of expressed UGTs to uncover the key conjugation enzymes and kinetic properties. Further, within-genotype variability would be very useful to capture in further liver bank studies, which probe enzyme function within and across genotypes. For the six alleles highlighted in Table 4, there are *in vivo* data to support the *in vitro* findings of altered activity, providing a measure of confidence that at least the nature of the effect (up- or down-regulation) is qualitatively correct.

**Table 4.** UGT polymorphisms with the greatest implications for human health risk assessment.

Allele	Impact on function <sup>1</sup>	Allele frequency	<i>In vivo</i> and molecular epidemiological evidence
1A1*6	30–70% ↓	<1% in African Americans and Caucasians 16% in Japanese	Allele associated with 2-fold ↑ in serum bilirubin and 15-fold ↑ in irinotecan toxicity
1A1*7	95% ↓	<1% in Caucasians 1.5% in Japanese	Increased risk of hyperbilirubinemia (Crigler-Najjar syndrome)
1A1*28	50–70% ↓	40% in African Americans and Caucasians 10% in Japanese	Increased risk of Gilbert's syndrome (unconjugated hyperbilirubinemia) and irinotecan toxicity
1A7*3	90% ↓	24% in African Americans 40% in Caucasians 16% in Taiwanese	Increased upper GI, colorectal and hepatocellular carcinoma. Increased irinotecan toxicity when combined with the upstream (T57G) SNP
2B15*2	50–80% ↓	55% in Caucasians	Decreased conjugation of lorazepam in one of two studies; decreased conjugation of endogenous male hormone
2B17 null	100% ↓	33% in Caucasians 35% in African Americans	Decreased conjugation and clearance of endogenous male hormones; decreased conjugation of tobacco-specific nitrosamine (NNAL)

<sup>1</sup>Change from reference phenotype in *in vitro* test systems with model substrates.

An important area of ongoing research is the manner in which numerous polymorphisms may be expressed in coordinated fashion to create haplotypes that have modified activity that can't be predicted from evaluating one SNP at a time. For example, the *UGT1A1*\*7 SNP occurs in the 5' end of the coding region and appears to influence cofactor binding to the enzyme. This SNP is common to multiple *UGT1A1* variants that generally have SNPs in other regions as well. However, the combined effect of *UGT1A1*\*7 and other *UGT1A1* SNPs is not known, and neither is the frequency of such haplotypes. This example illustrates the need to better characterize the frequency and effects of the combined or coordinated inheritance of influential SNPs.

#### 4. Hydrolysis with epoxide hydrolase

Microsomal epoxide hydrolase (*EPHX1*) is an enzyme found on the endoplasmic reticulum of many tissues and is responsible for the hydrolysis of various epoxides (Arand et al., 2005). Because epoxides are highly reactive oxidative metabolites, they are often the most toxicologically active form of a drug or environmental chemical. *EPHX1* breaks the three-membered epoxide ring structure by the *trans*-addition of water to form a less reactive diol that can then be conjugated and more readily excreted (Omiecinski et al., 2000). However, certain substrates such as the prototypic polycyclic aromatic hydrocarbon, benzo(a)pyrene, are activated by *EPHX1*; in this case diol formation is just one step in a sequence that leads to a second epoxidation and yields a highly mutagenic diol epoxide metabolite (Shimada, 2006). Therefore, *EPHX1* is sometimes implicated in xenobiotic activation. These chemicals can include arene epoxides (e.g., styrene oxide), polycyclic aromatic epoxides (e.g., B(a)P epoxide metabolites), alkane epoxides (e.g., ethylene oxide), and alkene epoxides (e.g., 1,3-butadiene, vinyl chloride, epichlorohydrin).

##### 4.1. *EPHX1* properties and function

*EPHX1* exists as a single copy on the long arm of chromosome 1. It is distinct from another microsomal epoxide hydrolase, cholesterol-5,6-oxide hydrolase, and from soluble epoxide hydrolase. The latter appears to play a role in modulating blood pressure, inflammation, and atherosclerosis, as it has been shown to hydrolyze epoxyeicosatrienoic acids, lipid signaling metabolites of arachidonic acid (Morrisseau and Hammock, 2005; Lee et al., 2006). Although the physiological role of *EPHX1* likely involves the detoxification of endogenous and exogenous epoxides, endogenous substrates are not well defined. It appears to be involved in sex hormone metabolism because the epoxidated steroid metabolites estroxiol and androstene oxide are both substrates (Vogel-Bindel et al., 1982). Its presence in rat epididymis suggests a role in testosterone production (DuTeaux et al., 2004). A role for *EPHX1* in the prevention of breast cancer via hydrolysis of estrogen epoxide has been hypothesized but this is only speculative (Newman et al., 2005).

*EPHX1* has been variously abbreviated *EPHX*, *EPHX*, and *EPOX*. It is a membrane-bound protein consisting of 455 amino acids, with a molecular weight of approximately 50 kDa (Newman et al., 2005). It has been found in nearly all mammalian tissues, with levels generally greatest in liver, followed by lung, heart, kidney, and testis. The gene consists of nine exons and eight introns with the coding region containing two relatively frequent SNPs, one in exon 3 and the other in exon 4. The exon 3 polymorphism contains a substitution resulting in histidine instead of tyrosine at codon 113 (Tyr113His; rs1051740, chromosome 1:22408622), whereas the exon 4 SNP involves arginine substitution for histidine at codon 139 (His139Arg; rs2234922, chromosome 1:224093029). These coding region SNPs have been extensively studied and will be the focus of the current analysis. Additionally, seven 5' flanking region polymorphisms have been identified that could theoretically modulate gene expression, but their influence is not well understood (Fretland and Omiecinski, 2000; Abdel-Rahman et al., 2005).

A concern with the interpretation of the available human studies is that *EPHX1* genotyping at the 113 codon contains a potential interference with a polymorphism at codon 119. A SNP at this location (Lys119Arg) can alter the generation of the restriction fragment length polymorphism (RFLP) in a manner similar to when tyrosine is replaced by histidine at codon 113. This accounted for 30% overreporting of the 113 SNP in a molecular epidemiology study of chronic obstructive pulmonary disease (COPD) patients when compared against other genotyping techniques that were used in conjunction with the standard technique (Matheson et al., 2006). An indication that codon 119 interference is occurring in the reporting of the codon 113 SNP is when this SNP is not in Hardy-Weinberg equilibrium in the control population (Gsur et al., 2003).

##### 4.2. Effect of SNPs on enzyme activity

Assessment of enzyme activity in relation to codon 113 and 139 SNPs has been carried out in recombinant *EPHX1* and microsomal systems (Maekawa et al., 2003; Hassett et al., 1994; Hosagrahara et al., 2004). The earliest genotype/phenotype study of *EPHX1* was conducted with the reference genotype (Tyr113/His139) and three variant (His113/His139; Tyr113/Arg139, His113/Arg139) constructs transfected into COS-1 cells, using benzo(a)pyrene-4,5-epoxide as substrate (Hassett et al., 1994). As shown in Table 5, protein level was adversely affected by the codon 113 Tyr to His variant (56% of reference), whereas the enzyme's intrinsic activity was slightly above the reference genotype. In contrast, the codon 139 polymorphism was associated with increased protein levels but decreased enzyme activity (Hassett et al., 1994) (Table 5). When a construct containing both SNPs was tested, the protein level was intermediate whereas intrinsic activity was close to that for the codon 113 SNP. Results from Maekawa et al. (2003) for five coding region SNPs (codons 113 and 139, and three others) tested with *cis*-stilbene oxide are also shown in Table 5. Only one SNP had a significant effect on *EPHX1* intrinsic activity (Arg43Thr) and one other,

His139Arg, had elevated protein level, this being consistent with the earlier finding of elevated gene product for the codon 139 SNP (Hassett et al., 1994). The codon 113 SNP had minimal effect in the Maekawa et al. system. All changes relative to the reference gene were 2-fold or less across these two studies.

In another genotype/phenotype analysis, both recombinant *EPHX1* protein and microsomes from reference genotype and variant individuals were assessed for activity towards *cis*-stilbene oxide and benzo(a)pyrene-4,5-epoxide (Hosagrahara et al., 2004). As shown in Table 6, the reference enzyme was up to 2 times more active than both the codon 113 and codon 139 variants, with some indication that the double polymorphism (His113/Arg139) creates a further lowering of enzyme activity. Results from microsomes were generally consistent, with up to a 2-fold change in enzyme activity, but these results were not statistically significant. The lack of a major influence of genotype on phenotype was also found when styrene-7,8-oxide was used as substrate (Wenker et al., 2000). A bank of 20 human livers was assessed for microsomal styrene epoxide hydrolase activity in relation to genetic variation within exons 3 and 4. A 3–5-fold variation in hydrolase activity was found but there was no correlation with genotype (Wenker et al., 2000).

**Table 5.** SNP effects on *EPHX1* levels as expressed in COS-1 cells (% reference activity).

Polymorphism	Location	Protein level	Intrinsic activity <sup>1</sup>	Reference
Tyr113His	Exon 3	56%	110%	Hassett, 1994;
		92%	105%	Maekawa, 2003
His139Arg	Exon 4	154%	81%	Hassett, 1994;
		128%	106%	Maekawa, 2003
His113/Arg139	Exon 3/Exon4	82%	106%	Hassett, 1994
Thr26Ser	Exon 2	103%	103%	Maekawa, 2003
Arg43Thr	Exon 2	110%	134%	Maekawa, 2003
Thr275Ala	Exon 6	118%	87%	Maekawa, 2003

<sup>1</sup>Substrate was *cis*-stilbene oxide for Maekawa et al. (2003) and benzo(a)pyrene 4,5-epoxide for Hassett et al. (1994).

**Table 6.** *EPHX1* genotype/phenotype data in recombinant and microsomal systems.<sup>1</sup>

Genotype	<i>Cis</i> -stilbene oxide	BaP 4,5-epoxide
Recombinant <i>EPHX1</i>		
Tyr/His (wt)	286 ± 10	57 ± 3.8
His113/His139 (113 variant)	158 ± 13.5	45 ± 7.0
Tyr113/Arg139 (139 variant)	178 ± 18.3	50 ± 5.3
His113/Arg139 (double variant)	146 ± 9.8	37 ± 2.2 <sup>2</sup>
Microsomes <sup>3</sup>		
Tyr/His (wt)	67.7, 52.5	9.24, 8.19
His/His (113 variant)	34.4, 33.6	7.68, 8.57
Tyr/Arg (139 variant)	41.5, 39.1	8.14, 9.53

*Note.* Results are specific activity (nmole/min/mg recombinant or microsomal protein).

<sup>1</sup>Data from Hosagrahara et al. (2004).

<sup>2</sup>*p* < .01

<sup>3</sup>Two microsomal samples from donors that were homozygous for each allele were analyzed for each genotype. None of the microsomal results were statistically significant.

These *in vitro* expression systems and microsomal assays support the concept that the common SNPs do not materially change protein function but may affect protein levels (Maekawa et al., 2003; Hassett et al., 1994; Hosagrahara et al., 2004). Evidence from *EPHX1* variants suggests lowered protein stability, as assessed by half-life measurements after inhibition of protein synthesis (Laurenzana et al., 1998). Although *in vitro* half-life determinations may not correspond well to *in vivo*, this system is a good way to compare across genotype. Differences in half-life for the reference gene product versus that produced by codon 113 and 139 SNPs were consistent with the functional studies shown in Tables 5 and 6, although the half-life differences were not statistically significant.

There are several inconsistencies in the data, such as the fact that the codon 139 polymorphism increases protein levels in some systems but decreased it in another (Hosagrahara et al., 2004). This, and the modest effect size seen across studies, indicates that a single *EPHX1* SNP is unlikely to have a major *in vivo* effect on *EPHX1* activity. The possibility that the same individual could carry the codon 113 reference sequence and the codon 139 variant, both associated with higher activity, has led to the following classification used in molecular epidemiology studies: low activity: codon 113 variant/codon 139 reference (113His/His/139His/His); medium activity: codon 113 reference/codon 139 reference (113Tyr/Tyr/139His/His); high activity: codon 113 reference/codon 139 variant (113Tyr/Tyr/139Arg/Arg) (Kiyohara et al., 2006a, 2006b).

Additional studies involving human samples analyzed *in vitro* or from *in vivo* pharmacokinetic studies have provided suggestive but not quantitative evidence for a genotype effect on phenotype. A liver bank study involving 40 human specimens (16 female, 24 male, 36 of the 40 were Caucasian, age range 7–63 years) found an 8-fold spread in *EPHX1* protein levels and activity (benzo(a)pyrene 4,5-epoxide as substrate). Only a fraction of this interhuman variability could be explained by codon 3 and codon 4 variants, with the remainder unexplained (Hassett et al., 1997). The polymorphisms did, however, appear to alter enzyme activity in the direction anticipated: the few samples homozygous variant at the 113 codon (His/His) and homozygous reference sequence at the 139 codon (His/His) had low activity relative to samples whose genotype lacked these features. However, the effect size was small (Hassett et al., 1997).

In a study of 58 Japanese epileptic patients who were taking carbamazepine, measurement of urinary ratios of carbamazepine epoxide to the *EPHX1* metabolites (diols and conjugated diols) was used to assess the functional significance of *EPHX1* polymorphisms within inherited groupings or haplotypes (Nakajima et al., 2005). Haplotype characterization stemmed from an analysis of 29 SNPs, 15 of which were found in coding regions and of these 5 actually produced amino acid changes in *EPHX1*. This included the well-characterized codon 113 and 139 polymorphisms. The SNPs were organized into three blocks relating to the part of the gene where they were found. Effects on *in vivo* enzyme function (carbamazepine epoxide hydrolysis) were

assessed separately for each block. Many different haplotypes and diplotypes were possible within each block due to the variety of SNPs identified, leading to a complex analysis. The 113 codon polymorphism was shown to be associated with significantly increased enzyme activity, whereas the 139 polymorphism was associated with decreased function. However, these changes were modest and opposite in direction compared with earlier *in vitro* studies with pure enzyme. Similarly, a liver bank analysis found 4-fold variation in *EPHX1*-mediated metabolism of carbamazepine epoxide, but this variation did not correlate with exon 3 or 4 polymorphisms (Kitteringham et al., 1996).

A far upstream promoter region designated E1-b has a major influence on the targeting of *EPHX1* to different organs (Liang et al., 2005). The location of this region is chromosome 1:222304957–222301977. A polymorphism in this region involving a double Alu insert of approximately 300 base pairs at positions –2214 and –1392 has been shown to decrease gene transcription in reporter assays in three of four cell lines into which the modified *EPHX1* was placed (Yang et al., 2009). These declines ranged from 10% to 40% relative to the noninserted reference gene. SNPs have also been identified in other upstream regulatory components, with seven of these SNPs analyzed for modification of gene expression (Raaka et al., 1998). These polymorphisms were organized into linkage units that correspond to broad regions of the *EPHX1* promoter. The coordinated expression of three SNPs in the –200 region did not affect gene expression, whereas the coordinated expression of three SNPs in the –600 region lowered expression by approximately 30% (Raaka et al., 1998). An unlinked SNP at –399 did not change gene expression. The implications of *EPHX1* regulatory sequence polymorphism on susceptibility to 1,3-butadiene mutations *in vivo* was assessed in lymphocytes from 49 styrene-butadiene plant workers (Abdel-Rahman et al., 2005). The –200 and –600 variant clusters were associated with elevations in mutations in the hypoxanthine-guanine phosphoribosyl transferase (HPRT) locus with the results for the –600 cluster statistically significant. This is consistent with the 30% reduction in gene expression seen in reconstructed systems for the –600 SNP cluster (Raaka et al., 1998). However, the information for this upstream SNP cluster is from a single *in vitro* study, with gene expression *in vivo* potentially modulated by numerous additional factors (diet, medications, age).

#### 4.3. Frequency of *EPHX1* variants

A number of genotyping and molecular epidemiology studies have assessed the frequency of *EPHX1* genetic polymorphisms at the codon 113 and 139 locations. The Kiyohara et al. (2006b) data set is a literature synthesis across numerous studies in each of the ethnicities shown in Table 7. The codon 113 His variant is relatively common across the populations analyzed, with allele frequencies greatest in Asian populations (51.2%), intermediate in Caucasians (30–40%), and least in African Americans (19.3%). The codon 139 variant allele is somewhat less frequent, generally in the 15–30% range,

with no clear distinction between ethnic groups (Table 7). Regarding the other (more than 25) SNPs, a study probing cell lines from Japanese subjects suggests that the other coding region SNPs (Table 5) have frequencies of 5% or less (Maekawa et al., 2003).

Transmission of the codon 113 SNP originally appeared to be inconsistent with classical Hardy-Weinberg calculations in that the homozygote variant frequency was greater than anticipated (Hassett et al., 1994). As described above, this may indicate inaccurate assay of the 113 SNP due to interference from a SNP at codon 119 (Gsur et al., 2003; Matheson et al., 2006). However, 11 of 13 studies incorporated into a meta-analysis of lung cancer risk showed Hardy-Weinberg equilibrium for the codon 113 polymorphism in control groups (Kiyohara et al., 2006b). This indicates that although the overreporting of codon 113 SNP is possible, many studies have avoided this problem. The codon 139 SNP pattern has matched Hardy-Weinberg expectations (Hassett et al., 1994; Kiyohara et al., 2006b). Linkage disequilibrium has not been demonstrated across variant alleles or across *EPHX1* and other genes.

The upstream double Alu insertion at positions –2214 and –1392 was probed in 450 frozen cell lines that represents a wide diversity of United States ethnicities. The homozygous double insertion trait (lower gene expression) occurred in 4% of the population, with the reference promoter (no insert) occurring in 60% of the cell lines (Yang et al., 2009).

#### 4.4. Other sources of variability in *EPHX1* activity

Rat liver *EPHX1* can be induced by exposure to a variety of agents, including certain carcinogens (nitrosamines, benzo(a)pyrene, 2-acetylaminofluorene, 3-methylcholanthrene, glycidamide), tetrachlorobiphenyls, and green tea extract (Yang et al., 2006; Fretland and Omiecinski, 2000). In fact, induction of *EPHX1* was the most sensitive index of glycidamide-induced modulation of gene expression in human epithelial cell cultures (Clement et al., 2007). The gene can be repressed by glucocorticoids via interaction with the upstream promoter. As described below, a number of chemicals have been shown to inhibit *EPHX1* activity *in vitro*, which has helped separate *EPHX1* function from

**Table 7.** Allele frequency of the major *EPHX1* SNPs.

Study/population	Number of subjects	Codon 113 SNP	Codon 139 SNP
Sarmanova, 2000 Czech Caucasians	416	His allele: 38.1%	Arg allele: 19.8%
Ada, 2007 Turkish Males	133	Genotype: Tyr/Tyr: 50.4% Tyr/His: 42.1% His/His: 7.5%	Genotype: His/His: 69.2% His/Arg: 28.6% Arg/Arg: 2.2%
Maekawa, 2003 Japanese cell lines	72	His allele: 36.8%	Arg allele: 32.6%
Kiyohara, 2006 Asian	191	His allele: 51.2	Arg allele: 13.8
Blacks	304	19.3	27.1
Caucasian	1456	33.8	18.7

other enzymes for which there is overlapping substrate specificity.

#### 4.5. Evidence of *EPHX1* modulation of toxicant metabolism

Numerous xenobiotics have an epoxide in their structure or are metabolized to one, conferring greater reactivity and toxicity. *EPHX1* catalysis of epoxide ring opening and hydration is a key step in the detoxification of these compounds, although in certain cases it is part of a multistep activation pathway. In the case of benzo(a)pyrene, the initially formed 7,8-epoxide is accessible to *EPHX1*, leading to enantiomeric diol formation. However, this can lead to a CYP-mediated stereoselective second epoxidation in the bay region (7,8-diol-9,10-epoxide). This yields a sterically hindered epoxide, which is resistant to *EPHX1* and thus has a greater ability to reach critical sites in DNA (Xue and Warshawsky, 2005). Benzene metabolic activation has been studied in reconstituted mixed-function oxidation systems in which purified enzyme components were used to show the role of different CYPs and other microsomal proteins in benzene metabolism (Snyder et al., 1993). The addition of *EPHX1* to this system yielded greater levels of hydroquinone, a key metabolite in benzene hematotoxicity and carcinogenesis.

Understanding of *EPHX1*'s role in xenobiotic metabolism has been facilitated by sequencing, cloning, and expression of the *EPHX1* gene, which has enabled reconstituted enzyme studies, site-directed mutagenesis, and the development of *EPHX1* knockout mice. The specific inhibitors 1,1,1-trichloropropene-2,3-oxide and cyclopropyl oxirane have also been used to elucidate *EPHX1*'s involvement in chemical metabolism. Knockout mouse studies have been particularly useful in demonstrating the importance of *EPHX1* in xenobiotic activation and detoxification *in vivo*. The *EPHX1*-null mouse was normal in terms of growth and reproduction, but was unable to form the carcinogenic diol-epoxide metabolite when dosed with 7,12-dimethylbenzanthracene (DMBA) and was resistant to DMBA-induced skin tumorigenesis (Miyata et al., 1999). An *EPHX1* knockout mouse model demonstrated the importance of this enzyme in detoxifying epoxide metabolites of 1,3-butadiene (Wickliffe et al., 2003). Knockout mice exposed via inhalation to 20 ppm were 5.4-fold more sensitive to genetic mutation as evidenced at the *Hprt* locus than mice with the reference genotype. Further, intraperitoneal (i.p.) injection of butadiene diepoxide demonstrated 4.8-fold more

DNA damage (comet assay) in the knockout mice (Wickliffe et al., 2003).

Table 8 provides a partial listing of drug and toxicant substrates for which *EPHX1* involvement has been demonstrated. Other substrates also metabolized by *EPHX1* are summarized elsewhere (Fretland and Omiecinski, 2000).

A variety of studies have evaluated the potential role of polymorphisms in exon 3 or 4 of *EPHX1* in modulating the metabolism and toxicity of known *EPHX1* substrates. Results have been inconsistent with *EPHX1* polymorphisms sometimes showing a significant effect on toxicokinetics and at other times not. Many of these studies also evaluated other enzymes involved in chemical metabolism, with interactive effects between *EPHX1* polymorphism and other enzymes sometimes noted. For example, polymorphism in *EPHX1* was found to influence the urinary metabolite profile in 250 Chinese benzene-exposed workers (Kim et al., 2007). In particular, catechol formation decreased in those polymorphic at the exon 3 (codon 113) locus, which agrees with the finding in some studies of this being a lower functioning enzyme. Catechol formation is a direct result of *EPHX1* action on benzene oxide. However, polymorphism at the exon 4 (codon 139) locus was associated with greater excretion of S-phenylmercapturic acid, the opposite of the expected result given that this is a metabolite not otherwise known to involve *EPHX1* and the codon 139 polymorphism has been associated with higher enzyme activity (see above). A study of 37 Italian taxi drivers failed to find an association between *EPHX1* polymorphism and urinary excretion of two benzene metabolites (S-phenylmercapturic acid, *trans,trans*-muconic acid). However, the taxi driver benzene exposure levels were considerably below those in the Chinese occupational study, which may have limited the ability to detect small shifts in metabolite profile (Manini et al., 2006).

These data with benzene exemplify the contradictory results for *EPHX1* polymorphism in human populations, as also seen for the following compounds:

- (1) Vinyl chloride (VC): high-activity genotypes were associated with higher VC-induced mutation in French workers, the opposite of expectations (Li et al., 2005); this effect was not statistically significant.
- (2) Aflatoxin B1: a Sudanese case-control study for hepatocellular carcinoma found low-activity genotypes associated with 2.6-fold more disease, which is the expected direction, but the result was not statistically

**Table 8.** Evidence for *EPHX1* involvement in chemical activation and detoxification.

Xenobiotic	<i>EPHX1</i> role	Type of evidence	Reference
Benzene	Activation	Purified enzyme	Snyder, et al., 1993
Benzo(a)pyrene	Activation	Enzyme inhibitor	Walters and Combes, 1986
7,12-DMBA	Activation	Knockout mice	Miyata, et al., 1999
Aflatoxin B1	Detoxification	Mutagenesis assay	Kelly, et al., 2002;
		Epoxide hydrolysis	Johnson et al., 1997
1,3-Butadiene	Detoxification	Knockout mice	Wickliffe, et al., 2000
Styrene oxide	Detoxification	Enzyme inhibitor	Chung, et al., 2006
Carbamazepine	Detoxification/inactivation	Recombinant enzyme	Eugster, et al., 1991

significant (Tiemersma et al., 2001); higher aflatoxin-albumin adducts were found in Ghanians polymorphic in codon 139 (higher-activity form, so opposite to expectation) and the effect was inconsistent between those sampled in 2001 versus 2002 (Dash et al., 2007); *EPHX1* was not associated with aflatoxin-albumin adducts in Gambians exposed via dietary exposure (Wild et al., 2000). However, aflatoxin B1 may not be a good test of the importance of *EPHX1* genotype because *in vitro* studies suggest that hydrolysis of the aflatoxin B1 epoxide occurs spontaneously with little enhancement from purified rat or human *EPHX1* (Johnson et al., 1997). This is in spite of the fact that *EPHX1* was shown to mitigate aflatoxin B1-induced DNA adducts and mutagenicity in several *in vitro* systems (Kelly et al., 2002).

- (3) 1,3-Butadiene: the low-activity *EPHX1* haplotype (H at codon 113, H at codon 139) is associated with greater HPRT mutant frequency in lymphocytes from workers exposed to high levels of 1,3-butadiene (Abdel-Rahman et al., 2003); however, two separate studies in cultured human lymphocytes exposed to the most reactive 1,3-butadiene metabolite (diepoxybutane) showed greater sister-chromatid exchange from those subjects who had SNPs conferring higher *EPHX1* activity (Schlade-Bartusiak et al., 2004; Laczmanska et al., 2006). The one study that has examined polymorphisms in upstream regulatory sequences and vulnerability to 1,3-butadiene mutation *in vivo* (49 styrene-butadiene workers in Texas) showed that the variant genotype in the -600 SNP cluster is associated with increased mutant frequency (Abdel-Rahman et al., 2005).
- (4) Styrene: there was no association between *EPHX1* genotype (high versus low activity) and lymphocyte cytogenetic damage in styrene-exposed workers (Teixeira et al., 2004), and *EPHX1* polymorphism did not predict the amount of styrene-induced DNA damage in human lymphocytes (Laffon et al., 2003). Probing of a human liver bank for *EPHX1* activity found substantial variability but this was not correlated with SNPs in the *EPHX1* gene (Wenker et al., 2000). However, others have found an association between *EPHX1* polymorphism and styrene oxide-hemoglobin adducts in styrene-exposed workers (Teixeira et al., 2007). Further, cytogenetic damage in a styrene-exposed worker group was correlated with *EPHX1* polymorphisms (Vodicka et al., 2001).
- (5) Benzo(a)pyrene: incubation of human lymphocytes with benzo(a)pyrene produced an increase in chromosomal aberrations, a response that was magnified in lymphocytes from donors who had higher *EPHX1* genotypes (Salama et al., 2001); further, lung cancer patients were evaluated for benzo(a)pyrene diol-epoxide protein and DNA adducts in relation to various polymorphic enzymes—slow *EPHX1* alleles were associated with less adducts (Pastorelli et al., 1998);

polycyclic aromatic hydrocarbon-exposed coke oven workers had greater DNA damage if they had a rapid *EPHX1* genotype in one study (Leng et al., 2004), but the opposite tendency occurred in other coke oven workers evaluated for HPRT mutant frequency (Viezzier et al., 1999); benzo(a)pyrene-DNA adducts in the lymphocytes of smokers was correlated with several high-risk metabolism genes but not with *EPHX1* polymorphisms (Lodovici et al., 2004).

These examples point out the difficulty in classifying *EPHX1* polymorphisms as conferring either low or high risk for particular chemicals or endpoints. This may in part stem from the small effect genotype appears to have on *EPHX1* phenotype, the fact that enzyme inducers or inhibitors can act independently to modulate enzyme function, and that other enzymes, in particular glutathione transferases, can also metabolize epoxides and thus mute the effect of *EPHX1* polymorphisms.

#### 4.6. Epidemiological associations with *EPHX1* polymorphism

The potential involvement of *EPHX1* coding region SNPs in the risk for human disease has been widely studied, particularly with regard to cancers that are related to inhaling tobacco smoke. Because *EPHX1* is involved in the detoxification of some tobacco-related carcinogens (e.g., 1,3-butadiene) and the activation of others (e.g., bay region polycyclic aromatic hydrocarbons, benzene), a common hypothesis has been that *EPHX1* coding region SNPs modulate the risk of smoking-related diseases such as lung cancer, other lung diseases, and a variety of other cancers (Kiyohara et al., 2006a, 2006b; Lin et al., 2006; Park et al., 2005a, 2005b; Lebailly et al., 2002).

##### 4.6.1. Lung cancer

The Tyr to His polymorphism at codon 113 was associated with a nearly 3-fold reduction in lung cancer risk in a study of 277 lung cancer patients and 496 controls (Gsur et al., 2003), suggesting that decreased *EPHX1* activity is protective (Gsur et al., 2003). The occurrence of the codon 139 polymorphism had no independent association with lung cancer in this study. However, high *EPHX1* activity genotypes at codons 113 and 139 were associated with a 2.3-fold increase in lung cancer incidence in 182 Caucasian lung cancer cases (Park et al., 2005a). This odds ratio rose to 4.7 when just considering lung adenocarcinoma. A study of 230 Finnish lung cancer cases is also supportive of low-activity *EPHX1* genotype being a protective factor, as the codon 113 polymorphism was associated with an odds ratio of 0.68, although the combined genotype analysis across codons 113 and 139 did not yield significant results (Voho et al., 2006).

The extensive literature in this area has been compiled into two meta-analyses (Kiyohara et al., 2006b; Lee et al., 2002). In the more recent analysis, 11 case-control studies involving 13 different ethnicities and other 4000 subjects were evaluated (Kiyohara et al., 2006b). When divided according to ethnic group, four of the studies found a protective effect

for the exon 3 low-activity variant (113 His/His), whereas in six cases there was no substantial association and three showed an increased risk with this genotype. Overall, the meta-analysis produced a summary odds ratio of 0.83 for the His/His variant, which achieved statistical significance only in Caucasians (0.65, 95% confidence interval 0.44–0.96) (Kiyohara et al., 2006b). The meta-analysis also evaluated the effect of the SNP at codon 139 (Arg/Arg), a modification that appears to increase enzyme levels. In this case, the studies were evenly divided between an increase and decrease in lung cancer risk, with the overall odds ratio not statistically significant. Thus, relative to the 139 SNP, a stronger case can be made for the importance of the 113 SNP in modulating lung cancer risk, as it may decrease *EPHX1* activity and the activation of carcinogens in cigarette smoke (Kiyohara et al., 2006b).

#### 4.6.2. Other lung diseases

The high oxygen content of the lungs also requires a high capacity for detoxifying oxygen radicals. *EPHX1* in lung is part of this defense system, with high-activity genotypes anticipated to be protective. *EPHX1* polymorphisms have been studied in relation to decrease in lung function during aging, as this is highly variable and likely has a genetic component. Lung function in a group of 591 smokers was followed for 5 years, with the group nearly evenly split into those whose functional decline was rapid and those resistant to the decline (Sandford et al., 2001). The odds for being in the rapid decline group were 4.9-fold greater for those who had a family history of chronic obstructive pulmonary disease (COPD) and the *EPHX1* 113 variant genotype (His/His). This suggests that the low-activity genotype may interact with other host susceptibility factors to affect aging and smoking-related declines in lung function. A similar result was found in 265 cotton textile workers in China whose lung function decline was accelerated by dusty workplaces involving exposure to endotoxin (Hang et al., 2005). The endotoxin-induced acceleration of lung function decline was 3 times greater in those with the slow *EPHX1* genotypes, both at the 113 and 139 codons.

COPD and other lung conditions have been extensively studied in relation to *EPHX1* polymorphism. Both COPD and emphysema were associated with slow *EPHX1* genotypes in groups of 50 to 90 disease cases in Edinburgh compared to nondiseased control groups (Smith and Harrison, 1997; Koyama and Geddes, 1998). The odds ratio was 4–5-fold elevated for COPD and emphysema but was not elevated for lung cancer or asthma in relation to *EPHX1* polymorphisms. Additional support for slow *EPHX1* genotypes being a risk factor for pulmonary disease was found in 187 smokers (or exsmokers) in whom the COPD incidence was independent of *EPHX1* genotype alone, but when considered in relation to GSTM1 and P1 polymorphisms, the *EPHX1* slow variant (His/His at 113) was associated with greater susceptibility in an interactive manner (Cheng et al., 2004). This suggests that certain toxicants in cigarette smoke may be detoxified by either system, with the influence of the *EPHX1* slow

genotype obscured if glutathione S-transferase (GST) activity is optimal.

These studies and those reported by others (Park et al., 2005b; Fu et al., 2007; Vibhuti et al., 2007) show a consistent association between the codon 113 polymorphism and increased susceptibility to COPD, whereas the codon 139 polymorphism (higher activity, theoretically less vulnerable) is less consistently associated with COPD. However, Matheson et al. (2006) did not detect a link between *EPHX1* polymorphism and COPD, with the positive results reported by others attributed to the fact that another polymorphism on exon 3 (replacement of Lys at codon 119) can make the RFLP/polymerase chain reaction (PCR) technique for the reporting of the codon 113 SNP less reliable. In their population, the 113 His allele was overreported by 30% when using the basic technique and they did not find an association between *EPHX1* polymorphism and COPD in 72 cases and 220 controls using improved genotyping methods (Matheson et al., 2006).

#### 4.6.3. Leukemia and lymphoma

*EPHX1* may modulate the metabolism of benzene and other cigarette smoke carcinogens that can cause cancer of blood elements. The limited literature in this area shows associations between rapid *EPHX1* genotype and increased vulnerability to leukemia and lymphoma, both generalized and non-Hodgkins (LeBailley et al., 2002; Soucek et al., 2002; Sarmanova et al., 2001). For example, acute myeloid leukemia associated with specific chromosomal aberrations was 4.4-fold more likely to occur in people who had the rapid 113/139 genotype (Lebailly et al., 2002). A study of 219 lymphoma cases found that the *EPHX1* rapid genotype was associated with a higher risk, particularly for non-Hodgkin's lymphoma (Soucek et al., 2002). However, the protective effect of the codon 113 SNP was greater in the heterozygous than in the homozygous variant case, which raises questions about the reliability of this finding.

#### 4.6.4. Colorectal cancer

Although *EPHX1* polymorphism has been evaluated with respect to a number of gastrointestinal tract conditions, perhaps the best studied is in relation to colorectal cancer. Colorectal cancer is associated with cigarette smoking and dietary exposures, with *EPHX1* having the potential to alter metabolism and risk from these exposures. A role for *EPHX1* polymorphism in modulating risk for colorectal cancer has been suggested by a number of studies. In a study of approximately 1400 Minnesota colonoscopy results, the odds of having a positive test were not affected by *EPHX1* genotype unless cigarette smoking and red meat consumption were also taken into consideration. In these cases, the association of the low-activity codon 113 polymorphism with higher risk of polyps was significant for those who smoked the most tobacco or ate the most meat (Ulrich et al., 2001). However, the opposite finding was reported in several studies in which the *EPHX1* high-activity genotype was associated with increased risk of colon cancer in relation to smoking or meat consumption

(Cortessis et al., 2001; Tranah et al., 2004; Huang et al., 2005). Still other studies failed to find a correlation between *EPHX1* genotype and colorectal cancer risk (van der Logt et al., 2006; Robien et al., 2005).

#### 4.7. Summary: Risk implications of *EPHX1* polymorphism and potential for distributional analysis

##### 4.7.1. Is the enzyme important to toxicant action?

Through a variety of genetic engineering techniques, *EPHX1* has been implicated in xenobiotic activation in certain cases but detoxification in others. Although this makes *EPHX1* a key consideration in the risk assessment of epoxides, *EPHX1* is typically not the only enzyme involved in a toxicant's activation. Other enzymes such as glutathione transferases may also be able to degrade the epoxide or *EPHX1* may just be one step in a multireaction pathway. Therefore, the implications of *EPHX1* polymorphism on toxicant action will likely be modified by the activity of other related enzymes.

##### 4.7.2. Are there influential SNPs of sufficient frequency to have a broad impact?

The Tyr113His and the His139Arg polymorphisms both appear to modulate protein stability, with the reference gene product having up to 2-fold greater half-life and enzyme levels in the case of the 113 locus. The opposite appears to be the case for the 139 polymorphism (variant with greater levels). However, these *in vitro* results were not reproduced *in vivo*, possibly because the effect on enzyme activity is modest and the ability to detect this change *in vivo* may be obscured by exposure to *EPHX1* inducers and inhibitors such as common carcinogens, green tea extract, and glucocorticoids. In spite of these difficulties, the literature generally considers the 113 reference/139 variant combination to be high-activity haplotype.

As shown in Table 7, the 113 variant allele is present in 20–50% of the population depending upon ethnicity, whereas the 139 allele is present in 15–30% of population. Homozygous variants have been found at a rate of 7.5% for the codon 113 SNP and at 2% for the 139 SNP. The codon 113 and 139 SNPs occur frequently enough to afford ample opportunity to study their implications *in vivo*. However, issues of study size come into play when trying to assess the “high-activity” haplotype (113 reference sequence, 139 variant) as is expected to occur in less than 1% of the population.

Upstream polymorphisms appear capable of modulating gene transcription to a modest degree, with the double Alu insertion at positions –2214 and –1392 present in the homozygous form in 4% of individuals. These individuals may have diminished *EPHX1* protein levels, although this suggestion is based upon *in vitro* expression systems only.

##### 4.7.3. Can the polymorphism inform health risk assessment?

There are too many uncertainties with regard to (a) genotype effect on phenotype; (b) the manner in which other factors (inducers, inhibitors) may modulate *EPHX1* activity and interact with or obscure the effect of the SNPs; (c) the complex

role of *EPHX1* in xenobiotic action given its potential both in chemical activation and detoxification. These relationships would ideally be well described in an integrated physiologically based pharmacokinetic (PBPK) model system that can predict the influence of *EPHX1* SNPs on internal dosimetry and risk. However, this does not appear to be feasible at the present time.

##### 4.7.4. Outstanding questions regarding the health risk implications of *EPHX1* polymorphism

Although the influence of *EPHX1* SNPs individually or collectively appears to be relatively small, these SNPs have been associated fairly consistently with altered risk for several diseases, including lung cancer and COPD. The exposures involved in these case-control ecological studies are unknown, although tobacco smoke was involved in creating elevated risks in some cases. Given the inconsistent effect of *EPHX1* SNPs on toxicant metabolism and the rather modest influence of SNPs on enzyme activity, the basis for these epidemiological associations is not readily apparent. This is an area that merits further exploration, along with the potential influence of upstream (noncoding region) SNPs on *EPHX1* gene expression.

## 5. Antioxidant cellular defense and xenobiotic detoxification: NADPH:quinone oxidoreductase 1 (*NQO1*)

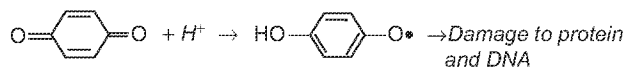
*NQO1* is a cytosolic enzyme that is widely distributed in mammalian tissues (Ross et al., 2000). It catalyzes the two-electron reduction of endogenous quinones and a variety of xenobiotics that can form quinone metabolites. Quinones are capable of forming stable adducts with cellular nucleophiles and undergo redox cycling that spawns reactive oxygen species. Therefore, the *NQO1*-mediated two-electron reduction of quinones is generally considered a detoxification reaction (Ross and Siegel, 2004). *NQO1* has received increasing attention because of this reductive capability in relation to well-known toxicants (benzene, ozone, chemotherapeutic agents), the vitamin K precursor menadione, and because the enzyme is overexpressed in tumor cell lines (Ross and Siegel, 2004; Rothman et al., 1997; Li and Yin, 2006; Bergamaschi et al., 2001). This section focuses upon a coding region polymorphism (*NQO1*\*2; rs1800566, chromosome16:68302646) that influences enzyme activity and is present at high frequency in various populations (Nebert et al., 2002).

### 5.1. *NQO1* properties and function

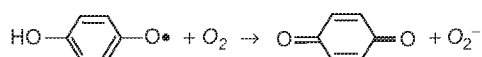
*NQO1*, also known as DT-diaphorase, is a dimeric flavoprotein of two equal subunits that uses NADH or NADPH to reduce a broad spectrum of electron-accepting substrates of endogenous and exogenous origin (Ross and Siegel, 2004; Vasilou et al., 2006). *NQO1* is characterized as a cytosolic detoxification enzyme due to its reduction of benzoquinones to hydroquinones. This two-electron reduction removes the prooxidant quinone and replaces it with the more stable hydroquinone. Quinones are an integral part of electron

transport chains, undergoing sequential reduction and oxidation as electrons are passed from donor to acceptor molecule via their electronegative oxygen atoms. Xenobiotics that form quinones can place an additional oxidant burden on the cell, as the quinones can accept a single electron, thus becoming a semiquinone radical. This partially reduced form can interact with oxygen to form reactive oxygen species that can damage the cell and at the same time regenerate the quinone moiety in a redox cycle sometimes termed a futile

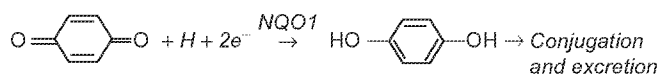
Reaction 1: Quinone to semiquinone via one-electron reduction



Reaction 2: Reformation of quinone and generation of superoxide



Reaction 3: Action of NQO1 to form hydroquinone and result in detoxification



cycle (Hochstein, 1983). Further, the semiquinone form is reactive in its own right and can bind to protein and DNA, leading to the potential for gene mutation and cancer as seen with benzene (Nebert et al., 2002), and to embryotoxicity as seen with diethylstilbestrol (Juchau et al., 1986) (Reactions 1 to 3).

NQO1 has broad substrate specificity for quinones and quinone-related compounds. It catalyzes a one-step, two-electron reduction that averts the semiquinone intermediate and thus ends the redox cycling of these molecules. The hydroquinone is relatively more stable and its hydroxyl substituents are good substrates for conjugation enzymes, which promote their excretion (Buffington et al., 1989).

NQO1 can act on quinones related to xenobiotics or generated from endogenous processes. The enzyme reduces oxidized forms of the coenzyme ubiquinone and  $\alpha$ -tocopherol, thus giving it a role in regulating cellular redox status, in preventing lipid peroxidation, and in the functioning of electron transport systems that involve ubiquinone (Landi et al., 1997; Siegel et al., 1997). Based upon these homeostatic and cellular defense functions, deficiency in NQO1 may be expected to predispose cells to a variety of chemical and oxidant stressors and lead to an increased susceptibility to cytotoxicity and tumorigenesis.

NQO1's biological functions have been explored in knockout mouse studies in which deficient mice were found to have reduced levels of circulating B cells, impaired primary and secondary immune responses, altered bone marrow redox status, and an increased tendency for myelogenous hyperplasia (Long et al., 2002; Iskander et al., 2006). NQO1 deficiency has been associated with increased hematotoxicity and leukemia

risk in humans exposed to benzene (Rothman et al., 1997). Although this may be related to NQO1's role in detoxifying quinones generated from benzene metabolism (Malik et al., 2006), the knockout mouse studies show that NQO1 deficiency may also predispose bone marrow to leukemia due to alterations in redox status and changes in immune function.

Although these effects may be related to the buildup of endogenous quinones, NQO1 has other biochemical roles that may contribute to these changes. NQO1 has been shown to regulate proteolysis of the P53 tumor suppressor gene product through its association with a soluble proteolytic system, the 20S proteasome. P53 is a highly labile protein, with a half-life as short as several minutes. Inhibition of NQO1 with the specific inhibitor dicoumarol led to decreased levels of P53 in human colon carcinoma cells and impaired the induction of P53 in response to  $\gamma$ -irradiation (Asher et al., 2001). This effect was reversed by co-incubation with protease inhibitors. Knockout mice also had decreases in P53 and decreased apoptosis in bone marrow cells (Long et al., 2002). NQO1's effect on P53 stability is consistent with the fact that the majority of this enzyme is in association with 20S proteasome, suggesting that NQO1 may modulate the degradation of numerous proteins (Asher et al., 2005). This may explain why the NQO1.2 protein itself has a short half-life and is highly sensitive to proteolysis (Ross and Siegel, 2004).

NQO1 is ubiquitous in bodily tissues, with particularly high levels in lung (Siegel et al., 1998; Bergamaci et al., 2001; Winski et al., 2002). It is noteworthy that whereas hepatic levels of NQO1 are high in rodents, they are relatively low in human liver. The enzyme is overexpressed in a variety of solid tumors, including those of pancreas, thyroid, breast, head, neck, liver, cornea, colon, ovary, and lung (Cullen et al., 2003; Winski et al., 2001, 2002).

Measurement of NQO1 in phenotyping studies has been limited to date, particularly in nontumorous normal tissues. Two quinone substrates have been used, menadione, a vitamin K precursor, and dichlorophenol-indophenol. These substrates are not specific to NQO1, but the anticoagulant drug, dicoumarol, is a specific NQO1 inhibitor. The combination of substrate and inhibitor provides a reasonably specific system for the measurement of NQO1 activity. There are no *in vivo* probe drugs for the phenotyping of individuals or populations. Instead, enzyme activity has been measured in human samples, such as cytosol from stored liver bank samples (Covarrubias et al., 2006) and from saliva (Siegel et al., 1999). Additional studies have been done in cultures of human tumor cells (Winski et al., 2002).

## 5.2. Effect of SNPs on NQO1 function

NQO1 belongs to the quinone oxidoreductase family, which consists of two genes, NQO1 and NQO2. Both genes are induced by a variety of environmental stressors, including oxidants in urban smog, PAHs, dioxins, and  $\gamma$ -irradiation, and appear to be part of a coordinated defense response that includes the induction of glutathione transferases, glucuronosyl transferase, and epoxide hydrolase (Jaiswal, 2000). Although NQO1 and NQO2 have similar function

and overlapping substrate specificity, they have different cofactor requirements (Long et al., 2002), and little is known about the endogenous substrates for *NQO2* (Celli, 2006; Jamieson, 2007). Additional studies are needed to determine the level of functional overlap between *NQO1* and *NQO2*.

The human *NQO1* gene is located on chromosome 16q22.1 (Ross et al., 2000). A total of 24 single nucleotide polymorphisms (SNPs) have been identified to date, with two SNPs, *NQO1*\*2 (rs1800566, chromosome16:68302646) and *NQO1*\*3 (rs1131341, chromosome16:68306370), being the best characterized (Ross et al., 2005). *NQO1*\*3 has been genotyped in human populations, with studies suggesting low occurrence (~1%) and reductive activity is reported to vary according to substrate (Ross et al., 2005).

In contrast, *NQO1*\*2 has clear functional significance and has allelic frequencies that exceed 50% in some subgroups (Ross and Siegel, 2004). It arises from C609T, which encodes a serine for proline substitution at amino acid 187. This polymorphism results in two changes that essentially lead to the null phenotype: (a) very low functional activity towards quinone substrates, and (b) low circulating levels of the protein. Diminished activity has been demonstrated in bacterial expression systems in which the variant form of the gene was introduced, with the resultant protein having 2–4% of the reference enzyme activity (Traver et al., 1997; Siegel et al., 1999). Diminished protein levels were demonstrated via immunoblotting in a number of normal tissues and tumor samples from individuals with the homozygous variant genotype (Siegel et al., 1999). This lack of *NQO1* protein apparently results from instability of the variant form as its half-life has been estimated to be only 1.2 hours in contrast to the reference enzyme (18 hours) (Siegel et al., 2001). Thus, a single key mutation in *NQO1* appears to both knock out activity and destabilize the protein, leading to essentially a null genotype.

The effect of genotype on enzyme function has received limited study to date. This is especially the case in normal tissues where two studies in humans have been reported, one in liver and the other in saliva (Covarrubias et al., 2006; Siegel et al., 1999). Somewhat more frequent are studies of enzyme levels in tumor tissues given the overexpression of *NQO1* in certain tumors and the implications for cancer chemotherapy (Ross and Siegel, 2004; Siegel et al., 1999). The largest study thus far in normal tissues involved human liver bank samples from 94 individuals, 31 African American and 63 Caucasian (Covarrubias et al., 2006). Although most specimens were from individuals with normal livers, there were some who had liver tumors or other disease; these individuals were not included in the current analysis of the reported data. Liver samples were genotyped and cytosols assayed for *NQO1* using the menadione/dicoumarol substrate/inhibitor combination. The other major cytosolic quinone reductase, carbonyl reductase (CBR), was also assayed and the *NQO1*-specific results were double-checked in a subset of samples by use of a CBR-specific inhibitor, rutin, which leaves only the *NQO1* activity. The authors report

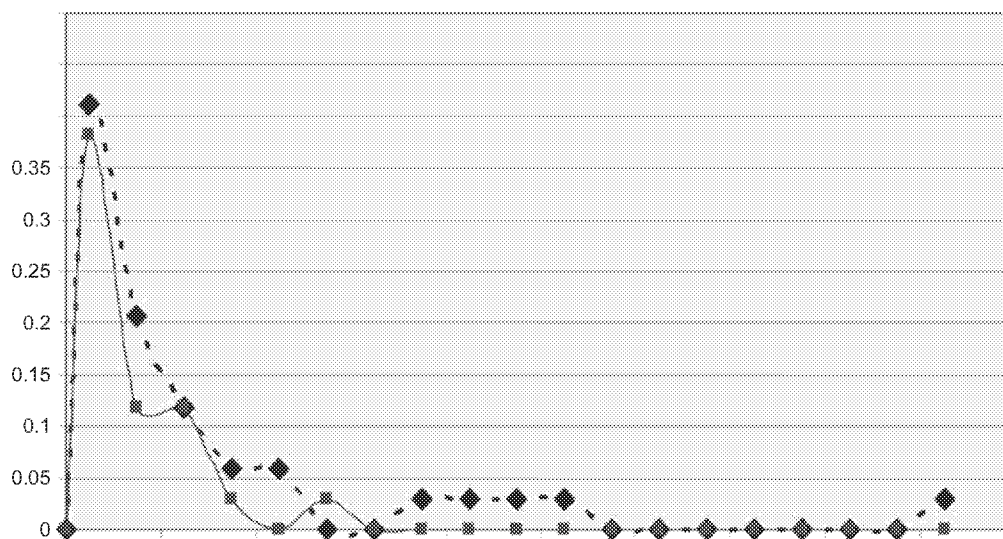
good correspondence between *NQO1* activity determined by both methods.

A significant ethnic variation was detected in which a 2.2-fold higher activity was seen in African Americans compared to Caucasians (Covarrubias et al., 2006); this interracial difference occurred regardless of genotype. Additionally, a statistical difference could be seen across genotype, as individuals with the reference genotype had liver cytosol *NQO1* levels that averaged more than twice those in heterozygotes in both Caucasians and African Americans. Surprisingly, the variability in enzyme activity for reference individuals was so large that approximately 50% of Caucasian reference individuals had nondetectable activity. An explanation for variability of this nature is not readily apparent, although it should be noted that the assay procedure involved subtraction of two different results (reductase activity with and without inhibitor), both of which involve their own degree of variability and measurement error which may obscure the results in low-activity individuals.

Expression of the *NQO1*\*2 allele is widely reported to produce little or no activity due to short half-life and low catalytic activity. It is noteworthy that Covarrubias et al. (2006) found *NQO1* activity to be nondetectable for one *NQO1*\*2/\*2 subject but clearly detectable in another, the latter finding being inconsistent with previous results in homozygous variant individuals (Siegel et al., 1999). It is possible that this individual experienced unusually high *NQO1* expression or decreased protein degradation. This subject had undergone glucocorticoid therapy and some steroid hormones may modulate *NQO1* activity. However, the influence of glucocorticoids on *NQO1* has not been evaluated.

Individual subject data were obtained from this liver bank study (J. Blanco, personal communication) and the enzyme activity frequency distribution was plotted for Caucasians according to genotype (Figure 6). Data for T/T individuals (*NQO1*\*2/\*2 genotype) were too limited for graphic presentation. The majority of individuals express relatively low levels of enzyme activity (less than 2 nmols/mg-min) in liver cytosols, representing approximately 82% of reference and 96% of heterozygous Caucasians. As shown in Figure 6, there was considerable overlap between the reference and heterozygote distributions in this low end of enzyme activity. However, reference individuals had a broader distribution, with several individuals having higher enzyme activity than what was found in any heterozygotes. A common feature for both African Americans (not plotted) and Caucasians is that none of the heterozygotes had activities above 4 nmol/mg-min whereas some individuals who with the reference genotype had activity well above 4 nmol/min-mg.

In another study evaluating the relationship between *NQO1* genotype and enzyme activity in human subjects, saliva was obtained from 27 individuals, 8 carrying the reference genotype (C/C), 8 with the C/T genotype, and 8 who were homozygous variant (T/T or *NQO1*\*2/\*2) (Siegel et al., 1999). Quantitation of *NQO1* protein in saliva via immunoblot analysis showed an approximately 50% reduction in heterozygotes relative to reference individuals, with no protein



**Figure 6.** *NQO1* activity distribution in Caucasians by genotype. Data from Covvarubias et al., 2006.

detectable in those who were T/T individuals. Although the difference between the C/C and C/T groups was statistically significant, there was a large degree of variability, approximately 4-5-fold, within each genotype. Analysis of specific activity found that the enzyme collected from C/C and C/T individuals had nearly equal activity, whereas no activity could be detected for T/T individuals.

The limited genotype-phenotype data available in normal human samples are supported by data from tumor tissues. NQO1 levels are up-regulated in a variety of tumors, suggesting that this enzyme confers a survival advantage for the proliferating cells. However, NQO1 can also be a disadvantage to tumor cells, as certain anticancer drugs such as mitomycin C may be activated rather than detoxified by the two-electron reduction catalyzed by *NQO1* (see below). In one study, phenotypic analysis of tumors found that reference individuals ( $n=14$ ) had 5 times greater NQO1 tumor activity than heterozygotes ( $n=5$ ) (Fleming et al., 2002). This intergenotype differential is larger than that found in normal human tissues, as described above (roughly 2-fold difference), but this may reflect some form of gene amplification or modified expression in the tumor cells. In general, tumors in individuals with the homozygous variant genotype lacked detectable NQO1 protein (Siegel et al., 1999; Zhang et al., 2003).

### 5.3. Frequency of NQO1 variants

Genetic variation in *NQO1* has been widely studied. The inheritance of the reference and deficient alleles are predictable based upon Hardy-Weinberg calculations (Ross and Siegel, 2004), with reported genotypes shown in Figure 7 (Rothman et al., 1997; Kelsey et al., 1997; Yang et al., 2007; Moore et al., 2004; Wang et al., 2006; Lincz et al., 2007; Lewis et al., 2001). There is marked ethnic variation in the occurrence of the deficient *NQO1*\*2 allele, with it being quite common in Asian populations where substantial percentages of the population (15–30%) carry two *NQO1*\*2 alleles and thus are considered null with respect to

enzyme activity. The null genotype represents 1–5% of the population in Caucasians and African Americans. The relatively high frequency of the null genotype in the Chinese population is a contributing factor to the prevalence of benzene hematotoxicity in the occupational studies described below.

#### 5.4. Other sources of variability in NQO1 activity

Many environmental factors can affect *NQO1* expression, including dietary components and environmental exposures that induce *NQO1* activity. These include isothiocyanates in broccoli, the common food preservative butylated hydroxyanisole (BHA), an ingredient in vegetable oil (conjugated linolenic acid), and the antihyperlipidemic drug and peroxisome proliferator clofibrate (Zhang et al., 2006; Hu et al., 2006; Bergamo et al., 2007; Moffitt et al., 2007). Substrates for the enzyme such as hydroquinone, a metabolite of benzene in bone marrow, have been shown to induce the enzyme, as well as other pro-oxidant exposures such as ozone and  $\gamma$ -irradiation (Jaiswal, 2000). Induction of *NQO1* in response to benzene is lost in those who have the variant genotype, which is likely part of the reason for greater sensitivity of these individuals to benzene hematotoxicity (Moran et al., 1999). Finally, *NQO1* expression is up-regulated in response to aryl hydrocarbon receptor (AHR) ligands, and together with CYP1A1, *NQO1* is an indicator of AHR activation. The classical AHR ligands 3-methylcholanthrene, beta-naphthoflavone and dioxin can all induce *NQO1* activity in rat liver (Brauze et al., 2006). The anticoagulant drug dicoumarol is an inhibitor used in experimental settings to ablate *NQO1* activity. Other factors may also impair enzyme function including tumor necrosis factor (TNF)- $\alpha$  and lipopolysaccharide (Gharavi and El-Kadi, 2007).

### 5.5. Role of NQO1 in modulating toxicity

There are a limited number of chemicals whose metabolic activation or detoxification has been associated with *NQO1*.

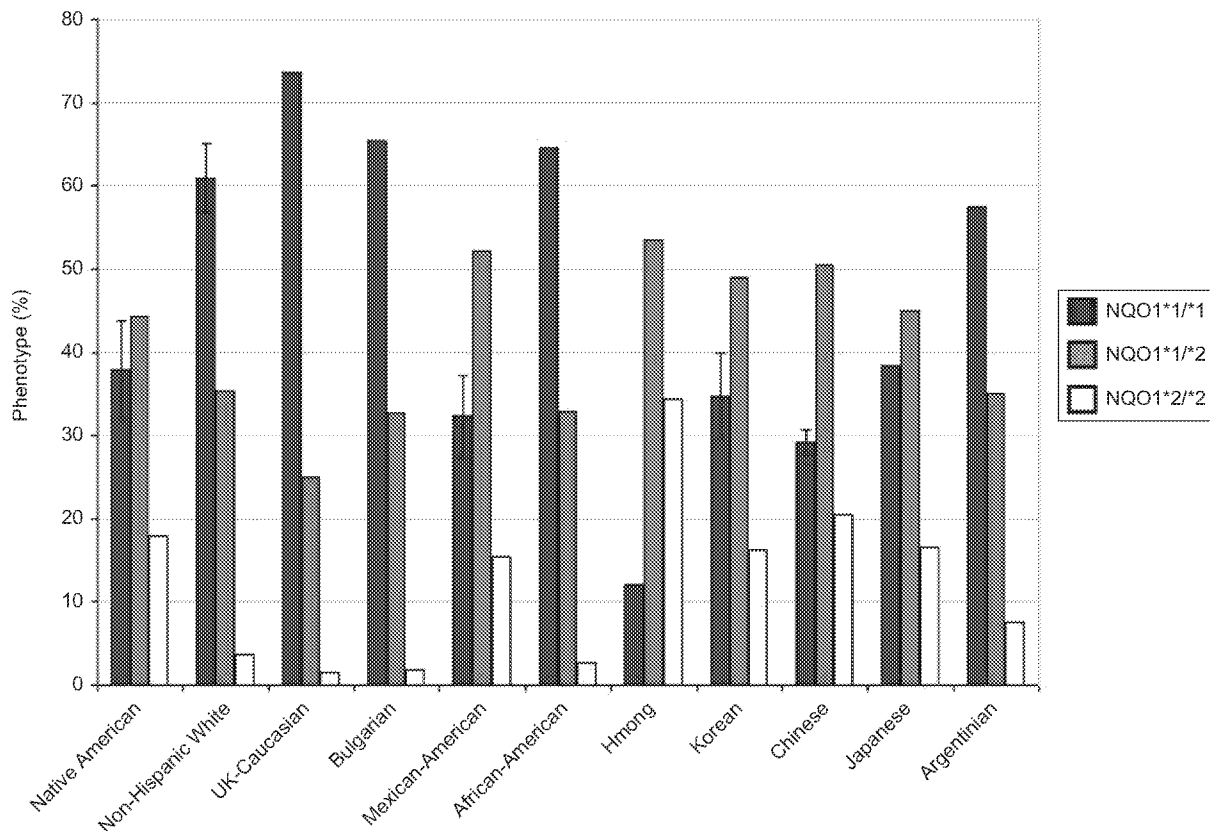


Figure 7. *NQO1* Genotype frequency across ethnic groups.

The major example to date has been benzene. Hematotoxicity and leukemia are induced by benzene via CYP2E1-mediated oxidation in the liver stemming from two sequential activation steps that yield hydroquinone. This metabolite is transported to bone marrow where it can undergo further oxidation to the more toxic benzoquinone and semiquinone radical (Nebert et al., 2002). Redox cycling of benzoquinone in addition to direct arylation of nucleic acids can form oxygen radicals and present toxic and leukemogenic risks to the bone marrow. *NQO1*'s role in benzene-induced hematotoxicity was studied in Chinese factory workers exposed to benzene at concentrations well above the US occupational standard of 1 ppm (Rothman et al., 1997; Nebert et al., 2002). Cases of benzene poisoning were defined as abnormally low white blood cell and platelet counts against a backdrop of benzene exposure with no other medical explanation for the lowered blood counts (Rothman et al., 1997). This syndrome was statistically linked to increased risk for developing leukemia and a variety of myelodysplastic syndromes in this population. A subset of these benzene-affected Chinese workers ( $n=50$ ) were genotyped for *NQO1* and *CYP2E1* in relation to 50 factory workers who were unexposed to benzene (Rothman et al., 1997). The odds ratio for benzene hematotoxicity was 2.7-fold higher in *NQO1*\*2/\*2 individuals compared to those who were heterozygous or homozygous for the reference enzyme, although this result was not statistically significant. This odds ratio rose to 7.6 and was statistically significant when the *NQO1*\*2/\*2 genotype

was present in association with high CYP2E1 activity as phenotyped with chlorzoxazone. Follow-up studies in 310 Chinese workers exposed to benzene and/or lifestyle cancer risk factors (smoking, alcohol) found that the *NQO1* allele was strongly associated with increased benzene risk, and that certain *CYP2E1* polymorphisms and the *GSTT1* null polymorphism increased the vulnerability to benzene toxicity (Wan et al., 2002). Oxidative damage to nucleic acids (RNAs) caused by occupational benzene exposure was enhanced in those subjects carrying the *NQO1* null allele (Manini et al., 2010). Overall, these results suggest a central role for *NQO1* in the detoxification of benzene quinone metabolites in organs such as bone marrow after CYP-mediated activation in liver.

Knockout mouse studies have also demonstrated the protective effect of *NQO1* on benzene hematotoxicity. When *NQO1* null mice were exposed to inhaled benzene concentrations of 10–100 ppm, 6 hours/day for 2 weeks, a 6-fold increase in genotoxicity was seen in females as compared to normal mice, as assayed by counting micronucleated peripheral blood cells (Bauer et al., 2003). Male mice were also affected but the pattern of response to benzene and modulation by *NQO1* differed somewhat across gender.

A detoxifying role for *NQO1* has been implicated in the skin carcinogenesis caused by PAHs (Long et al., 2000). *NQO1* knockout (null) mice who were treated with a single topical dose of benzo(a)pyrene soon went on to develop a graded dose-response for skin cancer in relation to amount

of BaP put on the skin. In contrast, mice carrying the reference genotype did not see any tumor increase at these doses. Follow-up studies demonstrated that the elevation of skin tumors in null mice was associated with benzo(a)pyrene 7,8-diol-9,10-epoxide rather than benzo(a)pyrene quinones, in spite of the fact that NQO1 is capable of detoxifying benzo(a)pyrene quinones (Iskander et al., 2005; Joseph and Jaiswal, 1998). This suggests that NQO1's protective effect was not due to a chemical-specific change in metabolism but rather by its demonstrated effects on cellular antioxidant status and responsiveness of the P53 system to induction by benzo(a)pyrene treatment (Iskander et al., 2006).

Contrary to the protective role NQO1 plays in benzene hematotoxicity, NQO1's reductive action has been shown to be essential to the toxicity of other agents. A variety of antitumor drugs are activated by one- or two-electron reductions, which gives NQO1 a role in their antitumor function. When colon adenocarcinoma cells devoid of NQO1 activity were instilled with the reference gene at various levels of incorporation, there was a direct correlation between NQO1 level and susceptibility to a variety of antitumor quinones (cytotoxicity, DNA cross-linking) (Winski et al., 2001). Similarly, in a study of patient survival on mitomycin C treatment for peritoneal cancer, better survival times were found if the tumors expressed the reference as opposed to the variant NQO1 allele (Fleming et al., 2002).

NQO1 plays a potential role in host susceptibility to ozone-induced cytotoxicity and inflammation in the lung. This was evaluated in 24 volunteers during a 2-hour exercise routine exposed to chamber concentrations of ozone ranging from 32 to 103 ppb (Bergamaschi et al., 2001). A variety of inflammatory and cytotoxicity indicators were monitored together with analysis of NQO1 and GSTM1 genotypes. There was a correlation between increasing ozone concentration and indicators of toxicity, which was most clearly in evidence in those with the NQO1 reference and GSTM1 null genes. This finding was attributed to the fact that NQO1 increases cellular levels of hydroquinones and ozone can directly interact with hydroquinones to yield the semiquinone radical as well as radical oxygen species. In this case, the NQO1-deficient genotype might be considered protective (Bergamaschi et al., 2001).

### 5.6. Epidemiological associations with NQO1 polymorphism

Evidence described above in knockout mice suggests NQO1 to have a key role in maintaining redox status in bone marrow and prevention of leukemia. This finding appears to have relevance to human leukemia risk, as shown in many but not all of the molecular epidemiology studies. Childhood leukemias are associated with a variety of different genetic abnormalities, but in the case of acute lymphoblastic leukemia (ALL) or acute myeloblastic leukemia (AML) in infants, there is often an MLL gene fusion (Wiemels et al., 1999). This gene defect was examined in 36 infant leukemia cases with documented MLL fusions versus 100 nonleukemic controls.

There was up to an 8-fold odds ratio for MLL gene fusion in association with the NQO1\*2 allele. In contrast, there was no association between NQO1 and other types of childhood leukemias. Further support comes from studies showing the NQO1\*2 allele associated with up to a 10-fold greater risk of ALL that was associated with MLL gene fusion in a small study of British infants (Smith et al., 2002), a 39% greater transmission of the NQO1\*2 allele in 657 childhood leukemia cases as compared to controls in Quebec (Infante-Rivard et al., 2007), and a greater incidence of ALL in Italian infants that in this case were not associated with the MLL gene fusion (Lanciotti et al., 2005). Not all studies showed such an association with childhood leukemia, as an investigation of 273 cases in Turkey showed no correlation of cases with NQO1 genotype (Sirma et al., 2004). Evidence for an association of NQO1\*2 allele with a variety of adult leukemias has also been found (Bolufer et al., 2007; Smith et al., 2001; Larson et al., 1999) although not in every case (Malik et al., 2006).

Overall, the evidence from both knockout mice and human leukemias suggests that NQO1 can protect bone marrow from endogenous oxidant stress and quinone buildup. Quinones have been shown to inhibit the DNA-processing enzyme topoisomerase II, an effect that may be related to the leukemogenesis caused by benzene and chemotherapeutic agents (Alexander et al., 2001; Hutt and Kalf, 1996). Therefore, a potential mechanism for NQO1 association with leukemogenesis is via the impairment of metabolic detoxification of chemical species that can inhibit topoisomerase II.

NQO1 polymorphism has also been studied in relation to a variety of other types of cancer, with a meta-analysis showing small elevations in risk for lung, bladder, and colon cancers in those with the NQO1\*2 allele, but this was an inconsistent finding across ethnic groups (Chao et al., 2006). Individual studies indicate that the odds ratio for colon cancer can be 2–3-fold elevated for homozygous variants, especially in association with alcohol and tobacco use (Begleiter et al., 2006). Several studies have shown associations between NQO1\*2 allele frequency and esophageal/gastrointestinal tumors in cohorts from Germany and China (Zhang et al., 2003; Sarbia et al., 2003). There is a suggestion that oral contraceptives may enhance the risk of breast cancer via an interaction with NQO1, as women who used oral contraceptives and later developed breast cancer were more likely to carry the reference genotype (Fowke et al., 2004).

### 5.7. Summary: Risk implications of NQO1 polymorphism and potential for distributional analysis

#### 5.7.1. Is the enzyme important to toxicant action?

NQO1 has been implicated as a key defense against quinone-mediated free-radical toxicity and carcinogenesis on the basis of (1) its ability to catalyze two-electron reductions; (2) knockout mouse studies show an increased vulnerability for a variety of adverse effects, including alteration of redox status in bone marrow in a manner that may predispose

to leukemogenesis; and (3) epidemiological studies have found associations between the null genotype and benzene-induced hematotoxicity, the action of anticancer drugs, and susceptibility to leukemia. These lines of evidence indicate the potential importance of *NQO1* in detoxifying certain quinones, activating others, and generally regulating cellular redox status.

### 5.7.2. Are there influential SNPs of sufficient frequency to have a broad impact?

The *NQO1*\*2 variant allele causes the gene product to degrade rapidly and what little protein is available has less than 5% of the activity as the reference gene product. This results in an essentially null genotype with a gene dosage effect for heterozygotes (intermediate activity). As shown in Figure 7, *NQO1*\*2 is broadly distributed across the general population, with this occurrence being greatest in Asian populations. This results in homozygous variant (null genotype) in 1–5% in Caucasians and 15–30% in Asians. These sizeable, genetically distinct groups have no *NQO1* activity and as such represent key populations for special focus in risk assessments involving toxicants whose mechanism of action involves quinones directly or that modulate levels of endogenous quinones by altering cellular redox status.

### 5.7.3. Can the polymorphism inform health risk assessments?

Useful information on genotype influence on phenotype is available as summarized above, but is somewhat uncertain. Figure 6 shows distributions of enzyme activity for Caucasians with either the reference or heterozygote genotype. This distribution can be combined with population genotype frequency shown in Figure 7 to simulate the population distribution of *NQO1* activity in Caucasians using Monte Carlo analysis. However, the genotype effect on phenotype data are limited and contain several uncertainties: large variability within genotype, small sample size, unexplained null activity in some reference individuals, and demonstrable activity in one null individual (Covarrubias et al., 2006).

### 5.7.4. Outstanding questions regarding the risk implications of *NQO1*\*2 polymorphism

A research priority is elucidation of the sources of phenotypic variability in *NQO1* given the unexplained intragenotype variability seen in reference and null individuals. Further, available evidence suggests important interethnic differences as African Americans had more than 2-fold greater enzyme activity than Caucasians for the same genotypes; however, there are less data for African Americans than for Caucasians (Covarrubias et al., 2006) and none for other ethnicities.

Additional uncertainties have to do with the role of *NQO1* in modulating toxicity. Relatively few agents have been identified whose toxicity is altered by *NQO1* status, although the list could theoretically be extensive. Greater use of knockout mice or null individuals to test hypotheses

regarding toxicant MOA is needed. As demonstrated with benzene and antitumor drugs, *NQO1* may increase or decrease toxicity and so the influence of the null polymorphism on toxicity will have to be evaluated on a case-by-case basis. Finally, there may be other enzymes or systems that can detoxify quinones such that risk may not strictly be a function of *NQO1* polymorphism and resultant activity level.

Ultimately, the implications of *NQO1* polymorphism on internal dose of quinones and related oxidative species (semiquinones, oxygen radicals) will require an integrated PBPK model that is parameterized for *NQO1* activity and can reflect the population distribution of activity in relation to underlying genotypes.

## 6. Overall summary

Our previous work documented the variability in enzyme activity caused by SNPs in several xenobiotic metabolizing enzymes (see Table 1 for references). The current effort extends those findings to four additional polymorphic enzymes evaluated for genotype effect on phenotype, frequency of influential alleles, and evidence of polymorphism effect on pharmacokinetics (clinical drug trials) and disease susceptibility (molecular epidemiology studies). These findings are summarized in Table 9. Although these enzymes are part of multigene families, each of which is associated with numerous polymorphisms, this effort highlights those enzymes and polymorphisms that are most clearly shown to modify xenobiotic fate or host defense. For example, of the six enzymes and 33 SNPs reviewed for the *SULT* gene family, only one SNP (*SULT1A1*\*2) is sufficiently well studied and has enough influence on enzyme function to receive a high priority for health risk assessment (Table 9). The other conjugation system analyzed, *UGT*, has SNPs in four different enzymes that merit consideration in health risk assessment due to their potential impairment of glucuronidation: *UGT1A1* (\*6, \*7, \*28 polymorphisms), *UGT1A7* (\*3 polymorphism), *UGT2B15* (\*2 polymorphism), and *UGT2B17* (null polymorphism). The *NQO1*\*2 (null) allele has the potential to impair host ability to reduce quinones formed endogenously or resulting from xenobiotic metabolism. This may predispose to various types of oxidative stress, radical damage, and susceptibility to specific toxicants that form quinones (e.g., benzene). In contrast, polymorphisms in *EPHX1* appear to be of relatively minor effect and thus are less likely to be of consequence in health risk assessments.

Although there are several clear examples of SNP effect on drug metabolism and toxicity (e.g., *UGT1A1*\*28 and irinotecan side effects; *ALDH2*\*2 and alcohol-induced gastric cancer), there have been few attempts to incorporate SNPs into human health risk assessment. The current review and prioritization is a necessary step for bringing SNPs into a population variability analysis that can be fed into risk assessment. However, these polymorphisms will only be relevant for those xenobiotics whose metabolic

**Table 9.** Implications of SNPs for human health risk assessment (HHRA).

Enzyme	Important to toxicant action?	SNP effect on enzyme activity	SNP frequency	Potential utility in HHRA <sup>1</sup>
SULT1A1	Many xenobiotics conjugated; is major SULT in human liver; however, other enzymes may overlap	*2: 2-10-fold ↓	30%	High
SULT1A2	Unclear since low expression in human liver	*2: >95% loss of activity	25-30%	Moderate, needs more data
SULT1A3	Less relevant for xenobiotics than for endogenous catecholamines	*2: 3-fold ↓ *3: 10-fold ↓	<5% <5%	Low Low
SULT1C2	Activation of <i>N</i> -hydroxyarylamines	*2: very little	≈5%	Low
SULT1E1	Conjugates endogenous and possibly environmental estrogens	*2: 90% ↓ *3 45% ↓	<5% <5%	Low Low
SULT2A1	Less relevant for xenobiotics than for endogenous hormones and bile acids	*2: very little *3: 45% ↓ *4: 85% ↓	15% 5% <5%	Low Low Low
UGT1A1	Major hepatic UGT involved in bilirubin and drug conjugation	*6: 50% ↓ *7: 95% ↓ *28: 70% ↓	15% Asian <5% 10-40%	High Moderate High
UGT1A7	Major GI tract UGT	*3: 90% ↓	15-40%	High
UGT2B15	Major stomach, breast, prostate UGT; conjugates estrogens, bisphenol A	*2: 50-80% ↓	55% in Caucasians	High
UGT2B17	Major g.i. tract UGT; acts on tobacco nitrosamines	Null: 100% ↓	30-40%	High
EPHX1	Detoxifies epoxides but can also lead to diol-epoxides	Tyr119His: <2× change His139Arg: <2× change	20-50% 15-30%	Low Low
NQO1	Detoxifies endogenous and xenobiotic quinones	*2: null activity	15-40%	High

<sup>1</sup>Utility rating based upon SNP effect on enzyme activity, SNP frequency, data availability for distributional analysis, and potential role in toxicant mechanism of action.

fate or mechanism(s) of action is affected by that particular enzyme. Further, there are various factors that can decrease the impact of a polymorphism, including the existence of enzymes with overlapping function and other pharmacokinetic factors (e.g., blood flow limitation) that can have a larger influence on metabolic fate (Kedderis, 1997; Lipscomb and Kedderis, 2002). Therefore, the full implication of the highlighted polymorphisms needs to be evaluated in physiologically based pharmacokinetic (PBPK) models.

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## Declaration of interest

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# Susceptibility Based Upon Chemical Interaction with Disease Processes: Potential Implications for Risk Assessment

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**Abstract** Numerous host and environmental factors may modulate vulnerability and risk. An area of increasing interest is the potential for chemicals to interact with background aging and disease processes, an interaction that may yield cumulative damage, altered chemical potency, and increased disease incidence. We evaluate the interactions possible between chemicals and background disease and identify the type of information needed to evaluate such interactions. Key among these is the existence of a clinically relevant and easy to measure biomarker of disease risk which is also modulated by a particular chemical of interest. This biomarker may be a physiological, biochemical, or genetic indicator that corresponds to a phase of the disease process and indicates where an individual is on the continuum between health and disease. The impact of toxic chemicals on this biomarker can then be used to predict how the chemical modifies disease risk, with this evidence strengthened by additional toxicology and epidemiology data showing toxicant effect on the disease process. Several case studies are presented which describe the toxic chemical, the clinical biomarker, the impacted disease and the evidence that the chemical enhances disease risk: fine particulate matter/decreased heart rate variability/increased cardiopulmonary events; cadmium/decreased glomerular filtration rate/increased chronic kidney disease; methyl mercury/decreased paraoxonase-1/increased cardiovascular risk; trichloroethylene/increased anti-nuclear antibody/autoimmunity; dioxin/increased CYP1A1/hypertension. These case studies point out that consideration of how a

chemical interacts with background aging and disease processes may increase the public health relevance of risk assessment, identify important vulnerabilities, and provide new ways to calculate risk from exposure to environmental toxicants.

**Keywords** Risk assessment · Vulnerable subgroups · Particulate matter · Heart rate variability · Cadmium · GFR · Mercury · PON1 · Trichloroethylene · Anti-nuclear antibody · Dioxin · CYP1A1 · Autoimmunity · Cardiovascular disease · Kidney disease · Hypertension

## Introduction

Risk assessment of chemicals has become more concerned with sources of inter-individual variability in recent years as the wide range of host and environmental factors that could potentially affect biological response have been explored. This includes differences related to life stage (fetal, childhood, adult, elderly), gender, lifestyle (exercise, sleep, diet), genetic variation, medications, co-exposure to other chemicals as well as non-chemical stressors, and the existence of endogenous damage processes that lead to disease [1–4]. The idea of cumulative risk assessment is thus being expanded to take into account not only multiple exposure pathways and similarly acting chemicals, but also the range of host-specific factors that may modulate the response to chemical agents. Facilitating this expansion of cumulative risk assessment is the increasing mechanistic understanding of chemical toxicity such that the instigating events (e.g., receptor binding, protein denaturation, DNA damage, lipid peroxidation, other forms of oxidant stress) and the downstream intermediary steps (e.g., genomic and epigenomic expression, disruption of homeostasis, involvement of inflammatory mediators) can be understood to occur to different degrees across the population, depending upon the host factors listed

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above. This paper focuses upon one particular variability factor, the occurrence of disease or pre-disease conditions in the host. As the upstream events that underlie chemical toxicity are compared with the endogenous damage events that underlie disease, their intersection is becoming recognized along with the potential implications for chemical risk assessment. This risk assessment focus was a key recommendation of the National Academy of Sciences as it reviewed the risk assessment methods of the US Environmental Protection Agency (USEPA) in the report referred to as *Science and Decisions* [4].

Perhaps the most compelling example of the interaction between an environmental agent and background disease is the contribution of fine particulate matter (PM <2.5  $\mu\text{M}$ ) to the risk of acute cardiopulmonary mortality. Fine PM can deposit in the deep lung and add to the burden of inflammatory stimuli that promote respiratory dysfunction and cardiac stress. Population studies have repeatedly found a linear or log-linear-appearing dose–response curve between fine PM and cardiopulmonary mortality in time series or cross-sectional (across cities) studies [5]. The most plausible explanation for this linearity in response at environmental doses common in the general public is that there is a broad range of vulnerability. Those already having compromised lung function are likely to be at particularly high risk and thus make up the group of low-dose responders [4]. Thus, one implication of toxicant–disease interaction is the continuation of the dose–response curve down to low dose when those with the disease represent a quantitatively important high-sensitivity subgroup. One way to identify such individuals is through upstream biomarkers of disease risk for which certain values (or percentiles of the marker distribution) are known to be indicative of higher disease risk. In the case of fine PM, a pre-disease biomarker of cardiac stress, heart rate variability (HRV) is both a predictive biomarker of risk for major cardiac events and an endpoint that is affected by fine PM [6–8]. While the mechanism(s) for this PM effect are still under investigation, it appears that PM-induced decrease in HRV is a key physiological response that contributes to the imbalance in autonomic control of heart rhythm, underlying, at least in part, cardiovascular morbidity and mortality. The implication is that chemical risk may be expressed as the projected increase in disease through an understanding of how the chemical modulates biomarkers that are predictive of disease risk. This may be particularly relevant at low doses where the chemical on its own might not be able to induce sufficient harm for a measureable outcome but may be a significant contributing factor to the occurrence of disease in susceptible individuals and thus increase population risk for that disease.

### Types of Toxicant–Disease Interactions

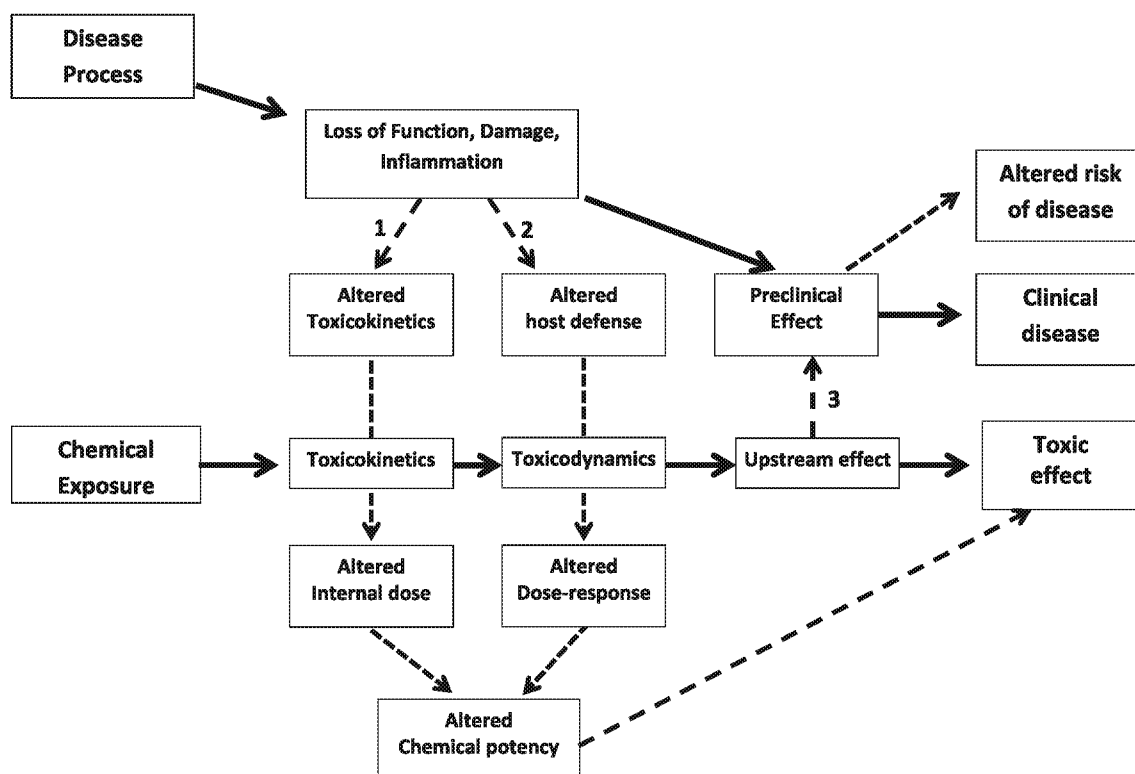
Background disease processes may affect vulnerability to toxicant action, while, at the same time, exposure to the toxic

chemical may affect the likelihood of disease in a given individual. The manner in which this can occur is described in the following three possible interactions and as outlined in Fig. 1 [9]:

- 1) The disease alters chemical action by altering its toxicokinetics so as to materially change internal dose. For example, the diabetic state is associated with higher levels of circulating ketones, which induce a particular cytochrome P450 (CYP) called CYP2E1 [10, 11]; this enzyme is known to be involved in the metabolic activation of numerous toxicants (e.g., trichloroethylene [TCE]).
- 2) The disease weakens host defense mechanisms, as could occur when a pathologic condition is associated with chronic inflammation and oxidative stress.
- 3) The chemical increases the likelihood of disease by altering pathways that are also affected by the disease process.

In the first two interactions, the presence of the disease process in the exposed individual can be expected to shift the dose–response curve to a lower level dose because the chemical is more effective in the diseased individual. A disease/dose additivity model may be useful in assigning ‘dose equivalents’ to a particular level of disease. In simple quantitative terms, if 5 mg/kg/d of a chemical decreases a disease biomarker by 20 %, while aging or a pre-disease state decreases the same biomarker by 10 %, one might conclude that the background condition contributes the equivalent of a 2.5 mg/kg/d dose. Thus, the starting point for chemical dose response is envisioned to not be zero but some dose equivalent (2.5 mg/kg/d in this case) based upon the level of disease with which it is interacting. An example of this phenomenon occurs with the hepatotoxicity data for the chemical 1,4-dioxane [4]. As shown in Fig. 2, the liver inflammatory response to 1,4-dioxane the pathology term is (hepatic spongiosis) differs greatly between male and female rats, and this corresponds with the gender difference in background rate of spongiosis. The high background of liver inflammation in male rats is additive to the 1,4-dioxane effect. Thus, the defense mechanisms in female rats that act as a barrier to 1,4-dioxane liver toxicity are exceeded in male rats, even in the control animals. In effect, it is as if the background condition is adding chemical dose equivalents such that the starting dose of chemical is already above its threshold for an adverse outcome, making the response at low dose linear in males and threshold in females.

In the third interaction, the chemical increases the likelihood for disease in the population by impacting on toxicity pathways that feed into the disease process. If a common biomarker can be found between disease risk and chemical effect, as in the case of PM affecting HRV, then the impact of the chemical on disease risk may be projected by the degree to

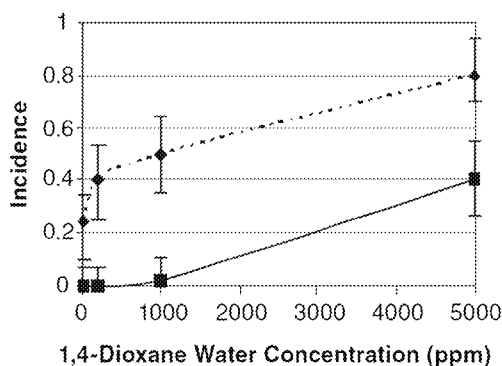


**Fig. 1** Potential interaction between chemical exposure and disease process. *Solid arrows* represent pathways of disease or chemical toxicity, which are typically considered as separate. *Dashed arrows* show potential interactions numbered as described in text. Interaction could lead to altered chemical potency as the disease process affects host toxicokinetics (Pathway 1) or defense mechanisms (Pathway 2), creating a vulnerability

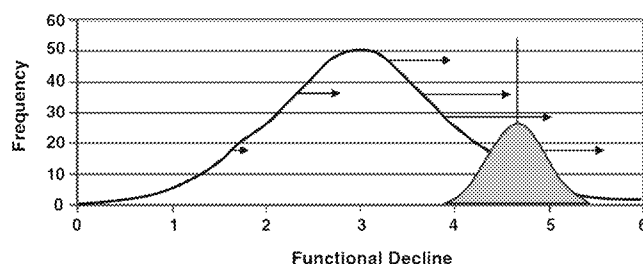
to chemical effect. Interaction could also affect disease risk, especially if the chemical and disease have similar upstream pathways and target organs (Pathway 3). In this case, chemical exposure creates an additional risk factor for disease to occur. Figure modified from the National Research Council report [9], with permission of National Academies Press, permission # 04301401

which it shifts the population towards poor function and increased disease risk. This is depicted in Fig. 3 [4], in which the population distribution for a continuous parameter becomes a vulnerability distribution if the parameter is a biomarker of human disease and if one can assign a degree of risk to a portion of the distribution. In the case of Fig. 3, functional decline increases along the X axis and crosses a point (vertical line) in which an overt adverse effect or clinical disease can be

recognized. The goal of medical management would be to keep individuals to the left of the clinical threshold, and the goal of risk assessment would be to understand how environmental factors (e.g., chemical and non-chemical stressors) might shift the distribution towards and over the clinical threshold. Thus, chemical risk may be evaluated as the percentage of the population that is shifted into the clinical disease portion of the distribution by a particular dose. This



**Fig. 2** Hepatic spongiosis in 1,4-dioxane-exposed rats: influence of control incidence on dose response. Bars indicate the 95 % confidence intervals. Data from Yamazaki et al. [12]; Figure from the National Research Council [4]. Reprinted with permission of National Academies Press, permission # 04301401



**Fig. 3** Population vulnerability distribution in the general case. Arrows represent hypothetical response to the same toxicant dose for people at a given level of functional decline unrelated to the toxicant. The vertical line represents the presumed threshold between overt disease and no adverse effect in median person. The shade area straddling the line represents the distribution of thresholds in the population. Figure from the National Research Council [4]. Reprinted with permission from National Academies Press, permission # 04301401

type of risk assessment (modification of disease vulnerability distribution) builds upon the experience with lead-induced shift of population intelligence quotient (IQ) distributions to lower levels, such that even small changes in mean IQ may have particular significance for individuals who are shifted into the low end (low functioning) tail of the IQ distribution [13, 14]. In this example, IQ can be seen as the upstream indicator of neurobehavioral success, as lead-induced IQ lowering has been used to estimate lost earning potential and poorer academic achievement [15]. This approach has recently been exemplified as described in subsequent sections for cadmium and methyl mercury [16•, 17].

To study toxicant–disease interactions in this manner, the following needs to be in place:

- 1) Underlying mechanism: evidence that the mechanism of toxicant action has aspects in common with the disease process (e.g., cellular targets, defense mechanisms, gene expression perturbations).
- 2) Disease biomarker: predictive biomarker of disease risk is identified for which the population distribution in healthy and diseased individuals (the vulnerability distribution) has been characterized. Shifts in the value of this biomarker are predictive of disease occurrence.
- 3) Chemical impact on biomarker: the chemical in question impacts the disease biomarker, in a manner which shifts the vulnerability distribution.
- 4) Chemical impact on disease: supportive epidemiological or toxicological evidence that the chemical can increase risk of the associated disease.

These toxicant–disease interactions are more likely to increase health risk if the rate of the background disease is substantial. With respect to Fig. 3, a rare disease will have a vulnerability distribution with most members of the population far to the left of the clinical threshold. A small shift in the distribution caused by a low dose of toxicant will cause very few (if any) individuals to cross into the clinical disease category – in that case we might say that there is low additivity to background disease. However, for more prevalent diseases, the vulnerability distribution would appear more like that depicted in Fig. 3, with a sizable fraction near the parameter value for clinical effect. In this case, numerous individuals would be sensitive to the toxicant, such that low doses may be able to shift a significant percentage into the disease tail of the distribution [15]. Thus, the increase in risk depends upon the dose response for chemical effect on the parameter and on the background rate of the disease as reflected in the shape of the vulnerability distribution.

The use of an upstream biomarker to evaluate the interaction of a toxicant with a disease process, as exemplified with

case studies described below, can be extended to epidemiological research. Studies that test the association between chemical exposure and adverse outcome can evaluate, through an interaction term or nested design, whether that outcome is more or less likely in those with a pre-existing condition (e.g., whether fine PM causes more severe effects in those with pre-existing lung disease). While this is useful, often the focus is on quantifying chemical exposure and not the degree of pre-existing disease, with the latter often considered in yes/no categorical fashion. Ideally, epidemiology studies would use continuous variables of disease risk (e.g., HRV) in association with continuous variables of chemical exposure to study their interactive effect on adverse outcomes. The case studies described in subsequent sections illustrate the utility of disease risk biomarkers in understanding chemical risk.

### Potential Role of Measurement Error

Before describing specific cases of chemical–disease interaction, it is useful to consider the role of measurement error in epidemiological studies. Evidence for toxicant interaction with background disease comes from epidemiology studies in which linear-appearing slopes exist down to low levels of exposure without evidence of threshold in spite of the likelihood that a threshold exists in individuals [4]. As the example with PM shows, where significant numbers of individuals have pre-existing disease that can be compounded by toxicant exposure, sensitivity and variability is introduced into the population dose response that tends to linearize the response at low doses. However, it has been argued that measurement error, rather than variability in response and interaction with background disease, is the reason why fine PM has a linear-appearing dose-response curve [18, 19]. Measurement error tends to smooth out or blur the dose response so that it can be more difficult to detect a clear break (non-linearity or threshold) in the dose-response curve. However, measurement error can also weaken associations and decrease the significance of regression coefficients such that those studies in which the regression coefficients are significant may be because they had less measurement error (or other confounders). There are other reasons to lessen emphasis on the measurement error explanation for linear-appearing dose response in population epidemiology studies.

A cornerstone of the measurement error hypothesis is the Brauer et al. [20] 2002 demonstration that errors in PM measurements are capable of washing out a built-in threshold in PM dose response for mortality. Brauer et al. [20] took personal monitoring measurements for 16 individuals for both PM and sulphate and compared these measurements with pollution measurements at regional monitors. The PM measurement error was large when relying on central monitoring locations, indicating that local and personal factors play an important role

in the value obtained. Brauer et al. [20] further demonstrated that the degree of measurement error in their PM data was sufficient to smooth out the dose response and obscure a threshold they had built into their dose-response model for PM-induced mortality [20]. Rhomberg and colleagues [18, 19] reviewed the cases of PM, nitrogen oxide, and ozone measurement variability with respect to the potential for measurement error to obscure thresholds in air pollution epidemiology studies. However, they do not consider the example of sulphate pollution, even though this is a key part of the Brauer et al. [20] study. In contrast to the PM results, sulphate measures from personal monitors correlated quite well with central outdoor air monitors across the 16 subjects. This led Brauer et al. [20] to show that, given the small measurement error for sulphate, it is easy to show a threshold in population studies should one exist (they simulated three different thresholds, each reliably reproduced for sulphate when the sulphate measurement error was built into the model). This suggests measurement error should not substantially mask population thresholds for sulphate or sulphur dioxide, for which total sulphate is a surrogate measure. By examining the population dose response for sulphate-related mortality relied upon by Brauer et al. (2002) [21], and an update of that in the World Health Organisation (WHO) guideline of 2005 [22], it is clear that many sulphate population studies are associated with linear-appearing dose response and that “As with ozone and PM, no obvious threshold levels have so far been identified in these population-based studies” [22]. According to the Brauer et al. [20] 2002 analysis, measurement error is unlikely to explain the lack of threshold for sulphur dioxide-increased mortality in the population studies reviewed by WHO. The sulphate example makes the case that factors other than measurement error can cause a linear dose response down to low dose in population studies for inhaled irritants. These other factors (e.g., inter-human variability) are summarized above for the general case.

Beyond air pollutants, numerous examples exist of population studies in which linear slopes occur down to low levels of exposure without evidence of threshold. In many of these cases, measurement error is unlikely to explain the lack of threshold since the index of exposure is biomonitoring data, i.e., quantitative internal evidence of exposure that does not rely upon extrapolation from job histories or external measurements. Examples include linear-appearing slopes for arsenic-induced cancer and non-cancer effects in relation to urinary inorganic arsenic [9]; for mercury-induced neurodevelopmental deficits in relation to maternal hair mercury [23, 24], which has been modelled as a linear relationship by Axelrad et al. [25] (2007); cadmium-induced decline in glomerular filtration rate (GFR), which has been found to have significant linear regression to low levels of urinary cadmium [26]; and lead-induced neurodevelopmental deficits

in relation to childhood blood lead [27]. Thus, while it is important to be aware of the potential influence of measurement error on dose response in human population studies, the examples mentioned above indicate that measurement error is unlikely to explain the linear-appearing slopes in many epidemiology studies and that it is important to recognize the role of background disease processes and other sources of intra-human variability in extending the response to low dose.

### Case Studies of Toxicant–Disease Interaction

As shown in Table 1, the elements for analyzing the toxicant–disease interaction are in place for at least three case studies (fine PM – discussed above, cadmium, and methyl mercury – discussed below) and are plausible for several others as discussed below. The biomarkers listed in the table facilitate exploration of these interactions, although their accuracy and link to clinical disease require assessment when considering their use in risk assessment.

#### Cadmium-Induced Renal Toxicity and the Risk of Chronic Kidney Disease

Kidney function progressively declines with age, as measured by various indices, including GFR [33]. A variety of conditions and diseases can put additional stress on the kidney, leading to increased risk of kidney disease. These conditions include diabetes, hypertension, obesity, cardiovascular disease, and systemic lupus erythematosus (SLE). The medical definition of advanced (stage 3 chronic kidney disease [CKD]) is based solely upon GFR: a GFR less than 60 ml/min/1.73 m<sup>2</sup> for at least 3 months is diagnostic of CKD. This GFR is well below the normal adult rate and is considered indicative of CKD regardless of age or the existence of other changes to the kidney [34]. Stage 3 CKD is clinically important, as it often leads to more serious renal impairment and the potential need for dialysis.

Certain environmental toxicants may interact with background aging and disease processes and jointly affect kidney function. Cadmium is a toxic metal that, by virtue of its known localization in the kidney, has this as a prominent target organ. Upstream oxidative stress signaling induced by cadmium (elevated mitogen-activated protein kinase [MAPK]-38 in renal cells) as well as clinical markers of cadmium toxicity (protein leakage into urine, decreased GFR) show that the effects of cadmium on the kidney are similar to that which occur from aging and disease [35–37]. The potential interaction of cadmium with background aging and disease processes has been assessed based upon modulation of the population distribution of GFR and the resulting increased risk of CKD [16••]. The potency for cadmium-induced decrease in GFR was derived from the regression slope observed in a study of

**Table 1** Chemicals, diseases, and biomarkers that can be used to evaluate toxicant–disease interaction

Chemical or physical agent	Interacting disease	Disease biomarker	Chemical impact on disease biomarker
Fine particulate matter	Cardiopulmonary mortality	HRV	Decrease in HRV [6]
Cadmium	Renal disease	GFR	Decrease in GFR [26]
Methyl mercury	Acute cardiac events	PON1	Decrease in PON1 [28]
Trichloroethylene	Autoimmune disease	ANAs	Increase in ANAs [29]
TCDD, PAHs	Hypertension	CYP1A1	Increase in CYP1A1 [30]
Styrene, ethylene oxide, radiation	Cancer, diabetes, heart disease, renal disease	CBMN	Increase in CBMN [31, 32]

*ANAs* anti-nuclear antibodies, *CBMN* cytokinesis-blocked micronucleus, *CYP* cytochrome P450, *GFR* glomerular filtration rate, *HRV* heart rate variability, *PAHs* polycyclic aromatic hydrocarbons, *PON1* paraoxonase-1

816 Swedish women chronically exposed to low environmental (rather than occupational) doses of cadmium [26]. This cadmium effect was applied to the GFR population distribution found in healthy adult women, resulting in a leftward shift of the distribution and inducing a greater percentage of the population to fall below the clinical definition of stage 3 CKD (60 ml/min/1.73 m<sup>2</sup>). The analysis showed that environmentally relevant cadmium doses of 0.1–1 µg/kg/d were estimated to increase the risk for CKD by 3–30 % [16••]. This example demonstrates that toxicant-induced risk of organ damage can be put in the context of population risk of chronic disease when the toxicant impacts an upstream biomarker that is predictive of chronic disease. This provides a quantitative method to assess the interaction of toxicant with aging and disease processes and thus may provide risk estimates of greater relevance to public health.

#### Methyl Mercury-Induced Oxidative Stress and the Risk of Cardiovascular Events

Cardiovascular disease is related to numerous risk factors ranging from genetic predisposition to dietary composition, activity level, stress and related disease states (e.g., metabolic disorder, thyroid imbalance, renal disease, and hypertension). In most cases, the potential role of toxic chemicals is overlooked. In the case of methyl mercury (MeHg), several epidemiology studies support an increased risk for cardiovascular disease, both in terms of narrowing of blood vessels and in increasing the risk of acute cardiac events [38–40]. However, not all epidemiology studies support such a link between MeHg and cardiovascular risk [41, 42]. This inconsistency may occur because MeHg exposure stems primarily from fish ingestion, and fish contain cardioprotective components, the most notable being omega-3 fatty acids [43, 44]. Therefore, the net cardiovascular risk or benefit associated with fish consumption is expected to vary depending upon which fish species and amounts are consumed by the population studied.

Further evidence of a cardiovascular risk associated with MeHg is the ability of MeHg to impair a cardioprotective

enzyme known as paraoxonase-1 (PON1). PON1 is associated with high-density lipoprotein (HDL) and prevents the oxidation of serum lipids and thus also the accumulation of oxidized lipid in vascular walls [45–47]. Its utility as a biomarker of cardiovascular risk has been demonstrated in several studies, including one in which 1,399 middle- to older-aged men were followed for 3 years after baseline serum PON1 measurements were taken [48]. The elevated risk of acute cardiovascular events in relation to baseline PON1 allowed the construction of a PON1 vulnerability distribution for cardiovascular risk [17]. Mercury has been shown to inhibit PON1 activity both in vitro and in vivo, with the in vivo study of a native fish-eating population in Canada demonstrating a dose response for lowering serum PON1 levels [28]. The mercury effect on PON1 likely stems from its attack on the lone sulfhydryl of PON1, a structural feature that is critical to its antioxidant properties. Application of the MeHg dose response for inhibition of serum PON1 to this PON1 vulnerability distribution led to a shift to lower levels of PON1 and to a greater number of individuals in the high-risk categories. This effect was accentuated when taking into account PON1 polymorphisms that lower the activity of the enzyme and thus have a compounding effect with MeHg on enzyme level and cardiovascular risk. Quantitation of the risk from an environmentally relevant MeHg dose of 0.3 µg/kg/d led to a projected decrease in PON1 by 6.1 % and an increase in population risk of acute cardiovascular events by 9.7 % [17]. Thus, reliance on mechanistic considerations and an upstream biomarker of cardiovascular risk enabled a quantitative investigation of the potential impact of MeHg on public health in a manner that complements the epidemiological evidence in this area.

#### Trichloroethylene-Induced Autoimmunity

Autoimmunity encompasses an array of progressive debilitating diseases that affect numerous organs, including the kidney, liver, thyroid, pancreas, gastrointestinal tract, skin, connective tissues, nervous system, and lung. The common link is inappropriate direction of the immune system against host tissues, which leads to chronic inflammation. This may result from the

modification of host proteins so that they become recognized as foreign, access to tissue components that are normally hidden but become exposed to immune cells because of cellular damage, or perturbation of immune regulation such that there is inadequate suppression of 'self-reactive' T and B cells [49, 50]. Exposure to chemicals may contribute to autoimmunity via these mechanisms, as some chemicals are capable of modifying host proteins to render them immunogenic [51], while other chemicals may damage tissues or alter gene regulation to affect the degree to which the immune system is self-reactive. Numerous pharmacological agents and environmental toxicants are implicated as promoting autoimmune conditions [52–54], with perhaps the best recognized of these being TCE [29]. TCE-induced autoimmunity has been evidenced in several mouse models, including one for SLE [55, 56], and in at least one strain not known for autoimmunity (B6C3F1 mice) [57]. Human studies also provide evidence of autoimmunity, as workers exposed to TCE had increased occurrence of scleroderma [58–60] and several other immune-related conditions [29]. The mechanism(s) for TCE-induced autoimmunity is unknown but may involve an initial toxic reaction in the liver involving TCE metabolism via CYP2E1 and subsequent oxidative damage and chronic hepatitis that stimulates the immune system to react against the liver and other tissues [61, 62]. Supplementation with the antioxidant sulfhydryl N-acetylcysteine was able to prevent TCE-related liver damage and autoimmunity [63]. This mechanism is likely to be operative at the high doses used in the Wang et al. [62, 63] studies (1,300 mg/kg via intraperitoneal injection) with this and perhaps other mechanisms occurring at lower doses where TCE autoimmunity has also occurred. TCE induces protein adducts in mouse liver, making it possible that the dichloroacetylated lysine adduct associated with TCE is a source of immunogenicity at low dose, although this possibility has not been fully explored [64]. While dose-response studies are thus far limited, the lowest dose found to enhance autoimmune parameters was 0.35 mg/kg/d in drinking water both in the mouse SLE model and in B6C3F1 mice [58, 65•]. This dose was associated with serological evidence of TCE-induced autoimmunity (anti-nuclear antibody [ANA] titers) with some evidence of renal involvement [58]. It also appears that inorganic mercury can enhance TCE-induced autoimmunity in autoimmune-prone mice, although the mechanism for this interaction has not been explored [66].

To better understand TCE-induced risk of autoimmunity at environmentally relevant doses, a biomarker of autoimmunity risk that is also sensitive to TCE would be needed. A potential candidate is ANA, since elevated ANA titer is a common finding in TCE studies in both mice and humans, with this effect extending down to the lowest doses tested [29, 65•]. Antibodies directed against nuclear elements and other proteins is a serologic marker in a variety of autoimmune

conditions, with this being used to assist early diagnosis of conditions such as type 1 diabetes and SLE [67, 68]. Thus, it is plausible that TCE-induced elevation of serum autoantibody markers can combine with genetic predisposition and inflammatory disease processes to elevate the risk of autoimmune conditions. Studies that further explore the dose response for TCE-induced increases in ANA and other autoantibodies in autoimmune disease models may help clarify the degree to which TCE increases the risk for these conditions in humans.

#### Dioxins and Other Aryl Hydrocarbon Receptor Ligands in Relation to Blood Pressure

Hypertension is a multifactorial condition that is highly prevalent in the human population. The fact that several epidemiological studies have shown a link between the expression of CYP1A1, a CYP isozyme that is regulated by the aryl hydrocarbon (Ah) receptor, and hypertension suggest that stimulation of the Ah receptor may contribute to the occurrence of hypertension. For example, epidemiological studies suggest that exposure to dioxin-like compounds is associated with higher blood pressure and that the precursor effect of the prototypical dioxin, TCDD, is ablated in knockout mice that lack the *CYP1A1* gene [69]. Further, polymorphisms in *CYP1A1* that modulate its inducibility and expression have corresponding effects on the risk for ischemic heart disease [70, 71]. The mechanistic link between *CYP1A1* expression and hypertension appears to be related to increased reactive oxygen generation as a byproduct of Ah receptor expression and *CYP1A1* metabolic processes [69, 72]. However, other aspects of Ah receptor activation by environmental ligands may also promote the hypertensive state as knockout mouse studies have shown the importance of the Ah receptor to maintaining blood pressure stability [73]. The contribution of Ah receptor ligands such as TCDD and polycyclic aromatic hydrocarbons (PAHs) to hypertensive and ischemic risk in human populations is becoming increasingly recognized [74, 75]. Thus, a potential biomarker of hypertension risk and chemical–disease interaction may be Ah receptor activation in the form of *CYP1A1* expression. It may be possible to screen the expression of, and polymorphisms in, this candidate biomarker in easily accessible samples such as peripheral blood lymphocytes (PBLs) as an indicator of the *CYP1A1* status of cardiac and endothelial tissues [76]. Further studies of the relationship between *CYP1A1* expression and blood pressure that control for the array of factors that can modulate blood pressure are needed to facilitate the use of *CYP1A1* as a marker of hypertension risk in the general population.

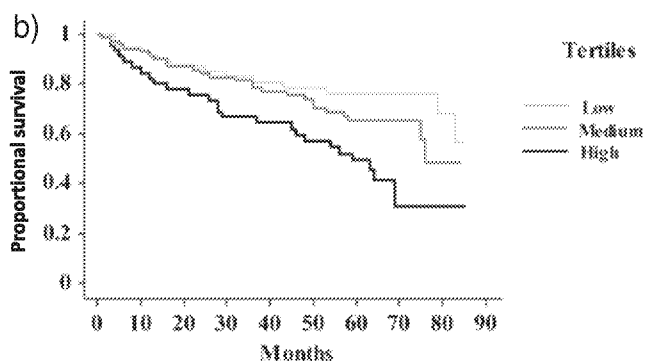
## Induction of Micronuclei by Pro-Oxidant Chemicals in Relation to Cancer and Inflammatory Disease

Oxidative stress is a result of inflammatory processes that underlie many common diseases. Exposure to pro-oxidant chemicals can add to the systemic level of oxidative stress and, at least in theory, also add to inflammation and disease burden. Oxidative stress can be biomonitoring in various ways, with the detection of micronuclei, a clastogenic result of oxidant attack on DNA, becoming increasingly common [32, 77]. Other agents that damage DNA via the formation of adducts or strand breakage can also lead to micronuclei formation, e.g., styrene oxide, ethylene oxide, residential radon, sunlight, and chronic alcohol intake [31, 78–81]. This form of DNA damage is readily seen in a cytokinesis-blocked micronucleus assay (CBMN) in which PBLs are arrested during cell division with cytochalasin B to freeze the cell in the binucleated state, during which it is easy to detect chromosome damage in the form of micronuclei. Increased rates of CBMN have been found in PBLs in association with age [82], and a meta-analysis of relevant epidemiology studies shows increased population cancer risk in individuals with moderately and highly elevated CBMN rates [83]. Other diseases are also marked by increased CBMN rates, including renal disease [84], type 2 diabetes [85], and cardiovascular disease [86•]. The predictive value of CBMN in determining cardiovascular risk is shown in Fig. 4, in which subjects with higher CBMN scores at the beginning of the study had greater rates of cardiac events and cardiovascular mortality upon follow-up [86•]. Thus, CBMN in PBLs may be a convenient marker of systemic oxidative stress and risk for a variety of chronic diseases. Given the wide variety of chemicals that can induce oxidative stress, CBMN may be a useful effect biomarker that can integrate across chemical and non-chemical stressors to

indicate cumulative oxidative stress. Identification of oxidant chemicals that can, at low dose, shift the CBMN population distribution to higher scores will help prioritize those agents that are likely to make a substantial contribution to the background rate of CBMN and thus affect population risk of chronic disease.

## Summary and Conclusions

The increasing focus on cumulative risk is helping risk assessors explore factors that would normally be treated with default uncertainty factors or not be addressed at all. An important variability factor concerns chemical interaction with background aging and disease processes. Disease processes that are prevalent in the population may confer a different vulnerability to toxicant action and may provide opportunities for toxicants to be active at low dose, levels below those representing a threshold in the average healthy individual. By developing a vulnerability distribution for disease occurrence based upon highly specific well validated clinically recognized biomarkers, it is feasible to explore how a chemical may interact with the disease process and shift the biomarker distribution to impact the risk of disease. The case examples involving cadmium/GFR and methyl mercury/PON1 provide quantitative examples of this approach, while the possibility for such analyses of TCE-induced autoimmunity and dioxin-induced hypertension may lead to better understanding of the public health risks from these chemicals. The CBMN example highlights that a non-specific biomarker of systemic oxidative stress may be useful as a cumulative index of disease risk and the contribution from individual pro-oxidant chemicals. To facilitate these and additional interaction studies, it will be critical for medical researchers, clinicians, toxicologists, and epidemiologists to jointly identify biomarkers of disease risk and explore how toxicants may perturb these biomarkers and thus modify population risk.



**Fig. 4** Predictive value of a cytokinesis-blocked micronucleus assay for cardiovascular survival. Kaplan-Meier curves showing cumulative rates of survival for cardiovascular mortality for major adverse events in patients with coronary artery disease according to micronucleus assay at baseline. Figure from Andreassi et al. [86•]. Reprinted with permission from Oxford University Press, license agreement # 3378870482866

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## Compliance with Ethics Guidelines

**Conflict of Interest** Gary L. Ginsberg, Rodney R. Dietert, and Babasaheb R. Sonawane declare that they have no conflicts of interest.

**Human and Animal Rights and Informed Consent** This article does not contain any studies with human or animal subjects performed by any of the authors.

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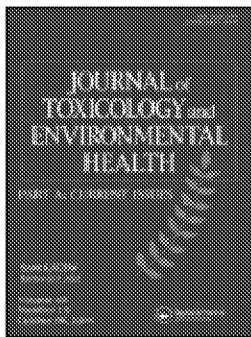
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# Modeling Approaches for Estimating the Dosimetry of Inhaled Toxicants in Children

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Risk assessment of inhaled toxicants has typically focused upon adults, with modeling used to extrapolate dosimetry and risks from lab animals to humans. However, behavioral factors such as time spent playing outdoors may lead to more exposure to inhaled toxicants in children. Depending on the inhaled agent and the age and size of the child, children may receive a greater internal dose than adults because of greater ventilation rate per body weight or lung surface area, or metabolic differences may result in different tissue burdens. Thus, modeling techniques need to be adapted to children in order to estimate inhaled dose and risk in this potentially susceptible life stage. This paper summarizes a series of inhalation dosimetry presentations from the U.S. EPA's Workshop on Inhalation Risk Assessment in Children held on June 8–9, 2006 in Washington, DC. These presentations demonstrate how existing default models for particles and gases may be adapted for children, and how more advanced modeling of toxicant deposition and interaction in respiratory airways takes into account children's anatomy and physiology. These modeling efforts identify child-adult dosimetry differences in respiratory tract regions that may have implications for children's vulnerability to inhaled toxicants. A decision framework is discussed that considers these different approaches and modeling structures including assessment of parameter values, supporting data, reliability, and selection of dose metrics.

## INTRODUCTION

The level of injury produced by inhaled toxicants depends upon the dose received by the lungs and internal organs. This dose is a function of numerous factors including (1) type of inhaled material, (2) region of the respiratory tract affected, (3) individual's ventilation rate, (4) type of breathing (oral vs. nasal), and (5) anatomical features such as airway diameter, branching pattern and regional surface area (USEPA, 1994).

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A number of these host-specific factors (e.g., airway architecture and ventilation rate) vary with age such that children generally inhale more air per body weight and respiratory tract surface area than adults (USEPA, 2002; Foos, et al., 2007). This can lead to child-adult differences in delivered dose, elimination, and toxicity. Models that estimate children's dosimetry are needed in assessing children's inhalation exposure and risk. Such efforts may point out whether higher delivered dose is a reason that children appear to be particularly sensitive to inhaled particles (Schwartz, 2004; Ha, et al., 2003) and gases (Gent, et al., 2003).

While our main focus is upon dosimetry in the respiratory tract, it is important to recognize that some inhaled gases are not extracted into tissues of the nose or conducting airways but penetrate distally to the pulmonary region where systemic absorption takes place. Due to greater ventilation rate and immature metabolism in young children (Makri et al., 2004), there may also be child-adult dosimetry differences for inhaled toxicants that are systemically absorbed (Nong, et al., 2006). Therefore, both local and systemic dosimetry may need to be simulated in children's inhalation models depending upon the nature of the inhaled material.

Fortunately, there are a variety of models available to simulate inhalation dosimetry of particles and gases and these models are generally adaptable to children's physiologic and anatomical parameters. However, due to data gaps for critical parameters such as airway architecture, available models are limited in detail and may miss important local areas of high deposition that may differ across age groups. Further, there are few datasets for the calibration or verification of children's models, particularly for young children where child-adult differences are expected to be greatest.

This paper summarizes presentations from an inhalation dosimetry session that was part of a two-day workshop on children's inhalation exposures and risks sponsored by the U.S.

EPA (June 8 – 9, 2006, Washington, DC). The session described research to adjust default modeling approaches for children's inhalation parameters, included a discussion of more detailed models for inhaled particles and gases and a presentation of a decision analytical framework for evaluating children's dosimetry models. The presentations are summarized in Table 1 and in the following sections. An introductory section describing general principles of respiratory dosimetry as affected by anatomical factors and the type of inhaled material is included. These principles provided the scientific basis and background for the workshop.

## REGIONS TARGETED BY INHALED TOXICANTS

Figure 1 shows a simplified representation of the respiratory tract divided into broad regions that receive deposition from different categories of inhaled materials. These regions have been demarcated based on major differences in size, structure, and function (U.S. EPA, 1994). These differences in turn exert dramatic effects on dosimetry in each region. The most proximal region, the upper respiratory tract, also referred to as the extra-thoracic region (ET), consists of the nose, larynx and pharynx. Large particles having a mass median aerodynamic diameter (MMAD) of  $\geq 10 \mu\text{m}$  impact on the walls and bifurcation points of the ET (U.S. EPA, 1994). This region is coated by mucus which protects the epithelium from gas absorption (and can alternatively result in toxic reaction products) or transport deposited particles out of the respiratory tract. This latter pathway results in secondary absorption in the GI tract. Smaller particles ranging in size from 2.5 to  $10 \mu\text{m}$  penetrate beyond the ET to the trachea and bronchi, referred to as the tracheobronchial (TB) region (U.S. EPA, 1994). These

conducting airways decrease in diameter and have an increasing number of branch points with increasing distance from the trachea. There are approximately 15 branch points or generations between the trachea and the pulmonary region, with the mucus coating progressively thinning with distance. Particles depositing in this region are typically cleared by physical dissolution or mucociliary action, or are transported to the interstitium via the lymphatics (ICRP, 1994). The relative contribution of these various clearance or defense mechanisms depends on particle size and distribution which also influences the location of initial deposition. For example, mucociliary clearance is less efficient in the deep bronchioles where dissolution and lymphatic clearance dominate. As described below, some models of respiratory tract deposition divide the human TB region into the upper bronchi extending to generation 8 (BB) and the lower bronchioles (bb) that range below generation 8 to the terminal bronchioles. These bronchioles have the capacity for gas exchange with the blood, but most of this exchange occurs in alveoli in the pulmonary (PU) region. Particles below  $2.5 \mu\text{m}$  and above the nano-size range are most able to penetrate to the PU region (Cheng, 2003). Clearance in this region may be by macrophage ingestion, lymphatic drainage, or in some cases dissolution. Particles have greater potential to induce adverse effects in this region due to longer retention and because macrophage ingestion may initiate an inflammatory reaction. The size cut points for deposition in the various respiratory regions are not absolute but provide a reasonable framework for understanding particle dosimetry.

This outline of respiratory architecture is also useful for describing where reactive (Categories 1 and 2) and non-reactive (Category 3) gases are extracted from the air stream (Figure 2). In 1994, the EPA introduced a categorization

**TABLE 1**  
Children's Inhalation Dosimetry Workshop Presentations

Speaker	Title	Material covered
Ginsberg	Application of default models to evaluate child/adult differences in regional and systemic dose	Default models for inhaled particles and gases run for 3-mo-old children and adults; includes regional dosimetry in lungs and systemic dosimetry for Category 3 gases as defined in U.S. EPA (1994).
Ashgarian	Particulate dosimetry modeling in the lungs of children	Multipathway lobar model run for ages 3 mo, 23 mo, 8 yr, 14 yr, 21 yr; clearance and retained dose also simulated
Ultman	Factors influencing the dosimetry of reactive gases in children	Diffusion-reaction model of reactive gas deposition including a mucus layer run for ozone at ages 0, 4, 8, 12, and 16 yr under varying activity levels
Kimbell	Nasal imaging and computational fluid dynamics-based dosimetry models	CFD modeling techniques as applied to children
Jarabek	The challenge to children's dosimetry modeling: creating a context for comparative analysis and consistent application in risk assessment	1994 RfC hierarchical framework for model structure evaluation; decision analytical framework for comparison of model structure, output, reliability and relevance to mode of action, variability and uncertainties

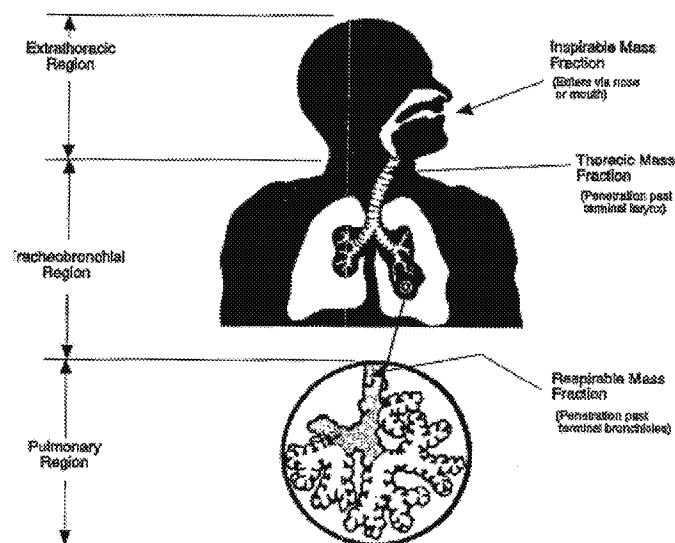


FIG. 1. Diagrammatic representation of three respiratory-tract regions. From U.S. EPA (1994).

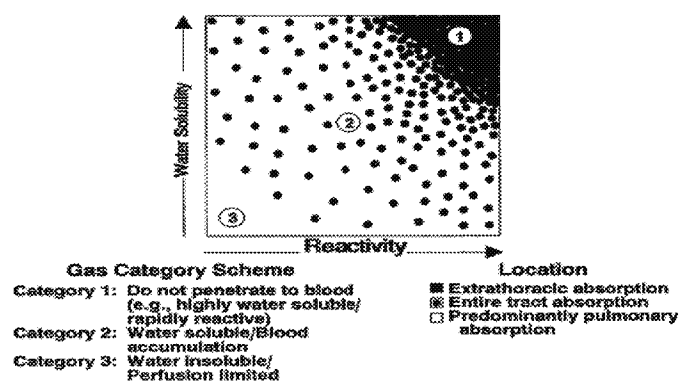


FIG. 2. Gas categorization scheme based on water solubility and reactivity as major determinants of gas uptake. From U.S. EPA (1994).

scheme for different types of gases that would help motivate different modeling approaches to describe dosimetry and arrive at dose estimates for each (U.S. EPA, 1994). The gas category scheme provides a framework for choosing an appropriate model structure that captures critical physicochemical properties of the inhaled gas and its interaction with physiological characteristics of the various respiratory tract regions. The goal is a description of the dosimetry of inhaled gases that is commensurate with the available data and level of detail regarding the mode of action for toxicity (Jarabek, 1995a). Although it should be recognized that the gas category scheme represents a continuum and that the same broad model structure may be applied to all categories, a review of the principles and properties informing category-specific default algorithms is helpful to understanding the dominant determinants of gas uptake in each region (Hanna et al., 2001). The framework motivated many of

the modeling efforts described herein for reactive gases in the upper respiratory tract (URT) (Andersen and Jarabek, 2001).

Category 1 gases (e.g., chlorine, formaldehyde, vinyl acetate) are either water soluble or reactive<sup>1</sup>, and are thus scrubbed out of the inhaled air primarily in the ET region at low exposure concentrations. Such gases typically exhibit a proximal to distal penetration and toxicity profile with increasing exposure concentration (U.S. EPA, 1994; Jarabek, 1995a). High levels of deposition in discrete regions of the nose combined with high reactivity leads to the potential for localized tissue damage (Morgan, 1994; Kimbell, et al., 1997). Only a small percentage of a Category 1 gas penetrates beyond the ET at low concentrations typical of ambient exposures, with this penetration greatest during exercise when ventilation rates are the highest (U.S. EPA, 1994; Nodelman and Ultman, 1999).

Category 2 gases are intermediate in reactivity and water solubility, which allows them to penetrate more readily beyond the ET and into the bronchi, and to a lesser extent the PU region. Some have the potential to accumulate in blood and thus have systemic as well as local effects, or may also deliver the toxicant back to the airway tissues from the endothelial side (U.S. EPA, 1994; Jarabek, 1995a). While not as reactive as Category 1 gases, Category 2 gases such as ozone ( $O_3$ ) still attack cellular constituents. Their potential to produce damage may be enhanced because they penetrate deeper into the airways where the protective mucus layer is thinner (Chang, et al. 1992; U.S. EPA, 1994). However, mucus is not always effective as a protective barrier since toxic reaction products can form in mucus and these penetrate to underlying epithelial tissue (Feng, et al. 1997). Category 3 gases, such as the chlorinated solvents chloroform and trichloroethylene, are non-reactive in the respiratory epithelium and not water soluble. They are not scrubbed out in the conducting airways but instead penetrate to the PU region where they are absorbed into the systemic circulation. Their toxicity is typically related to metabolic activation in liver and kidneys, or delivery of parent compound to the CNS.

This outline of particle and gas deposition points to the importance of the ET region in removing larger particles and reactive gases from the inhaled airstream. Air traversing the oral passages encounters less surface area than air that is inspired nasally, and toxicant removal in the nasal passages is more efficient due to smaller airway dimensions (Heyder, et al. 1975). Therefore, the dose that is available to the deeper airways is generally larger from oral as opposed to nasal breathing. Nasal breathing predominates at low to moderate ventilation rates but is augmented with oral breathing at higher rates that are associated with exercise and exertion (Niinimaa

<sup>1</sup> The U.S. EPA included in its definition of reactivity the ability of the inhaled gas to serve as a substrate for metabolism in respiratory tract tissues. For example, vinyl acetate, while not especially water soluble, is readily extracted in the URT via carboxylesterase metabolism and is considered a Category 1 gas.

et al., 1981; ICRP, 1994; Bennett et al., 2008). Given that children's activity and ventilation patterns are different than those of adults, it is possible that the oral-to-nasal ratio may be another age-specific factor that affects inhalation dosimetry.

The manner in which the various factors described above (particle size, reactivity of gases, ventilation rate, respiratory surface area, and oral-to-nasal ratio) affect inhalation dosimetry in children and adults are explored through various modeling techniques in subsequent sections.

## APPLICATION OF DEFAULT INHALATION DOSIMETRY MODELS TO EVALUATE CHILD-ADULT DIFFERENCES IN REGIONAL AND SYSTEMIC DOSE

Risk assessment of inhaled toxicants often involves cross-species extrapolation of an inhaled dose associated with an effect observed in a laboratory test species. Models used for this extrapolation range from rudimentary forms with a minimal number of parameters that accommodate sparse databases, to more sophisticated structures with detailed mechanistic descriptions of tissue responses (Jarabek, 1995b). The U.S. EPA (1994) provided a hierarchical and flexible framework for evaluating when alternate structures offered advantages to the default algorithms. Characteristics of models that would be considered "preferred or optimal" relative to "default" structures are shown in Table 2 (U.S. EPA, 1994). Considerations include whether the model utilizes chemical- and species-specific mechanistic information or rather relies on categorical, empirical parameters for key determinants such as ventilation and metabolic rates. For the default descriptions, the U.S. EPA's 1994 reference concentration (RfC) methods introduced rudimentary models that relied on predominantly empirical

descriptions of particle deposition and gas uptake, but nonetheless also represented reduced forms consistent with more sophisticated, detailed structures. For example, the gas-phase mass transfer coefficient used in the RfC methods is analogous to those used for models of O<sub>3</sub> and formaldehyde (Hanna et al., 2001; Kimbell et al., 2001a; Overton and Graham, 1989), and the inhalability adjustments and fractional deposition algorithms are analogous to those used in the ICRP and multiple-path particle dosimetry (MMPD) particle models described below. What distinguishes these models is the degree of detail and data underlying different descriptions (e.g., delineation of bronchioles and interstitial compartments in the ICRP model, localized gas flux estimates within the URT for formaldehyde uptake in the CFD models).

While this framework was useful in developing risk estimates for interspecies extrapolation from lab animal inhalation toxicology studies, the RfC methodology did not explicitly include adjustments to account for the physiological and anatomical differences that occur throughout a lifetime. Potential uncertainty in resultant risk estimates due to variability across life stages was believed to be addressed by the various uncertainty factors (UF) applied in operational derivation of the RfC including those specifically recognized for intrahuman variability and database deficiencies. However, recent emphasis on children's risk (Landrigan, 1999; FQPA, 1996) warrants exploration of modifying available dosimetry models to account for children's dosimetry directly, as well as for evaluating the adequacy of the methodology as a whole, including the intrahuman and database UF, in light of such simulations.

At about the same time, the International Commission on Radiological Protection (ICRP, 1994) developed a respiratory tract dosimetry model for the assessment of exposure from inhaled radionuclides. The ICRP approach is more inclusive than the RfC methodology by utilizing activity patterns for a wide range of children's age groups beginning with 3 months of age as input to the model. Further, as a semi-empirical model that includes some theoretical and mechanistic algorithms, the ICRP model includes explicit descriptions of various particle deposition mechanisms, e.g., particle impaction (based upon particle size and air velocity) and deposition via diffusion (based upon diffusion coefficient in air). Because the RfC methods rely on an empirical model description of deposition data that did not include particles of the diameter subject to diffusion as a dominant mechanism of deposition, the RfC model is not recommended for extrapolation outside that range (Raabe et al, 1988; U.S. EPA, 1994). As such, the ICRP model may provide a more reasonable model for the broader range of particle sizes with which to extrapolate to different ages based on varying ventilation rates, and may provide better estimates of regional inhaled dose than the regional empirical descriptions in the RfC methods. For example, the ICRP model divides the TB region into the more proximal portion (to generation 8) and the bronchioles (below generation 8).

**TABLE 2**

Hierarchy of Model Structures for Exposure-Dose-Response and Interspecies Extrapolation

### "Optimal" model structure

- Structure describes all significant mechanistic determinants of chemical disposition, toxicant-target interaction, and tissue response
- Uses chemical-specific and species-specific parameters
- Dose metric(s) described at level of detail commensurate to toxicity data

### Default model structure

- Limited or default description of mechanistic determinants of chemical disposition, toxicant-target interaction, and tissue response
- Uses categorical or default values for chemical and species parameters
- Dose metric(s) at generic level of detail

*Note.* Source: U.S. EPA (1994).

Inclusion of activity patterns and age-specific ventilation rates to calculate children's risk using the ICRP model was a critical component of the Agency's effort to update the National Ambient Air Quality Standards (NAAQS) for particulate matter (PM) in 1996, and similar simulation exercises with these activity patterns were performed with the MPPD model for the Agency's 2005 assessment and standard setting. Thus, exploration of using age-specific ventilation rates in other risk assessment arenas may benefit from a similar approach.

### GENERAL MODELING APPROACH

The default modeling approaches used to compare a 3-month old infant and adult respiratory tract dosimetry are depicted in Figure 3.

Particle deposition simulations using the default models and in the more refined modeling approaches described later in this paper estimate dose as the amount of toxicant delivered to a particular region per unit time normalized to surface area. The rate of clearance from that region to estimate retained dose was not considered. Thus, delivered rather than retained dose is described.

Since both the default RfC models and ICRP models were used to assess particle deposition, comparisons were made not

only across age groups but also across default models. For particles, deposition was either calculated from the equations in the RfC methodology or was taken directly from the deposition fractions provided by the ICRP model and then normalized to surface area to calculate the dose metric compared between the models. Runs of the RfC model are truncated for particle sizes below 1  $\mu\text{m}$ . These particles are subject to diffusion and are below the range in which this model was calibrated. The incorporation of children's parameters into these models represents scaling of the adult architecture and does not necessarily reflect an accurate comparison of airway structure or morphometry across life stages. For example, the ICRP model structure was adapted to that of a 3-month old infant by scaling and calculations based upon airway cast measurements as shown in Table 3. It was also run with adult parameters to compare estimates of delivered dose between children and adults. Three months of age was chosen as the mid-point for the first 6 months of life, a time when child-adult differences in ventilation rate per lung surface areas are likely to be greatest. The adult male parameters of the ICRP were used to calculate the adult estimates presented (ICRP, 1994).

Both the particle and gas uptake models were run under nasal breathing, light activity conditions to represent the most common breathing pattern, although it is recognized that

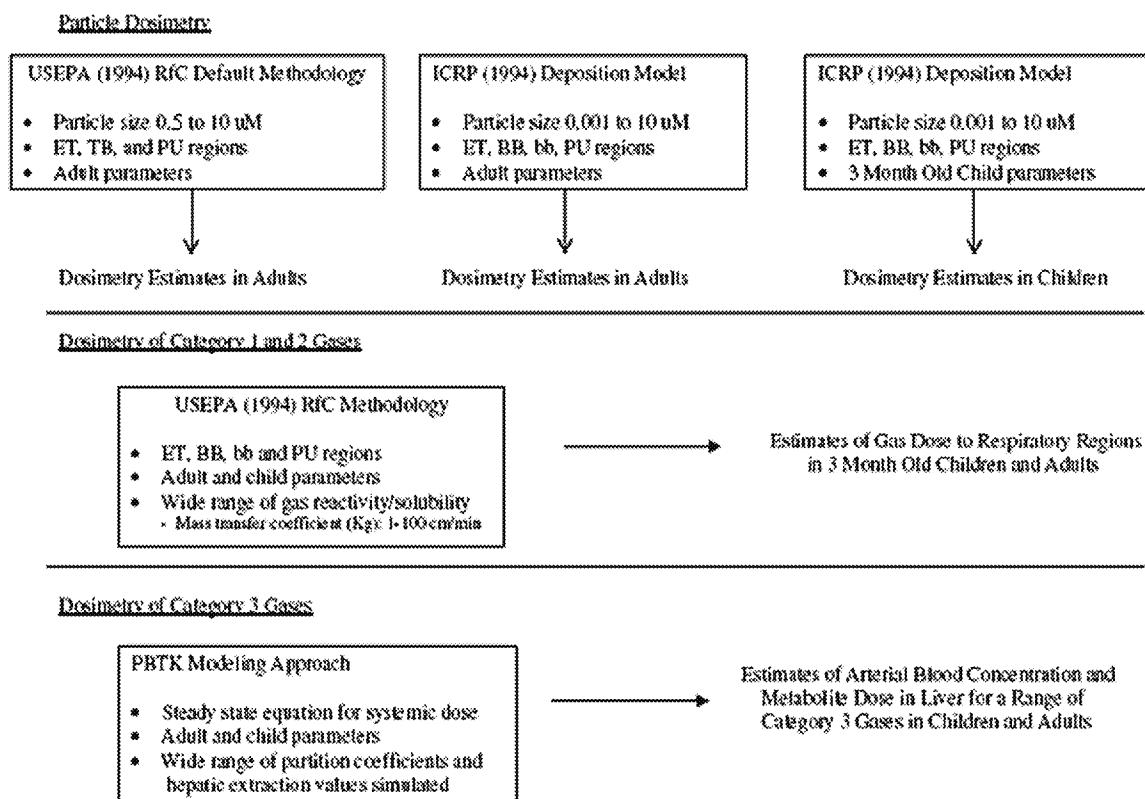


FIG. 3. Approach for dosimetry comparisons across default models in 3-mo-old infant and male adult. Modified from Ginsberg et al. (2005).

**TABLE 3**  
Regional Surface Areas (cm<sup>2</sup>) and Ventilation Rates in 3-mo-Old Infant and Adult Male

Region	Current analysis		Data source	RfC methodology <sup>a</sup>
	3 mo	Adult male		Adult male
ET <sup>b</sup>	40	160	Sarangapani et al. (2003) based upon age-related changes in cranial size from CT scans	200
TB <sup>c</sup> upper(BB) <sup>d</sup>	58	283	Phalen et al. (1985) scaling parameters and surface area calculations based upon surface area of a cylinder	3200 <sup>e</sup>
TB lower(bb) <sup>f</sup>	1512	2080	Phalen et al. (1985) scaling parameters as for TB region	— <sup>e</sup>
PU <sup>g</sup>	37704	777417	Yu and Xu, 1987 equations for alveoli number and dimension increase with age	540,000
Ventilation rate (ml/min)	2372	13800	Rate for light degree of activity (Harvey and Hamby, 2002; Sarangapani, et al., 2003)	13,800

Note. Adapted from Ginsberg et al. (2005).

<sup>a</sup> U.S. EPA (1994).

<sup>b</sup> ET = extrathoracic region of respiratory tract.

<sup>c</sup> TB = tracheobronchial region of respiratory tract.

<sup>d</sup> BB = bronchi generations 1–8 as per ICRP (1994).

<sup>e</sup> RfC methodology does not treat bronchi and bronchiolar regions as separate, so that the surface area shown for TB is the sum of both upper and lower bronchial regions.

<sup>f</sup> bb = Bronchioles: generations 9 and below as per ICRP (1994).

<sup>g</sup> PU = pulmonary region of the respiratory tract.

higher ventilation rates and switchover to oral breathing might lead to deeper penetration of particles and gases in both children and adults (U.S. EPA, 1994). A wide variety of particle sizes and gas reactivities were modeled in this screening exercise to evaluate whether certain combinations of toxicant properties and respiratory regions might lead to substantial child-adult dosimetry differences.

Reactive gas modeling for children (3-month old infant) and male adults was simulated for gases with a variety of reactivities by using a range for the overall mass transfer coefficient ( $K_g$ ) from 1 to 100 cm/min. This parameter is typically measured empirically in isolated regions of the respiratory tract and takes into account the net transfer of the gas due to convection and molecular diffusion in the air-phase, water solubility and chemical reactivity (e.g., hydrolysis), diffusion, and metabolism in the liquid lining and tissue phases, as well as clearance into the blood (Hanna et al., 2001; Andersen and Sarangapani, 2001). The  $K_g$  is thus an empiric measure of total flux from the air into the mucus or tissue layer and is both a species- and an age-specific parameter. This is related to the fact that convection (bulk flow due to ventilation) and diffusion thicknesses that govern  $K_g$  are dictated by airway architecture for the lumen and tissue.

The  $K_g$  is used to predict the amount of toxicant that actually interacts with cellular constituents and is often the key

input parameter to physiologically-based pharmacokinetic (PBPK) models that provide more detailed mechanistic descriptions of reactions within different tissue types as described in Section 5 below. Category 1 gases such as chlorine, formaldehyde, hydrogen fluoride, and organic acids and esters have  $K_g$  values in the upper end of this range and are scrubbed out of the airstream primarily in the ET region (Nodelman and Ultman, 1999; Overton, 2001; USEPA, 1994). Category 2 gases such as O<sub>3</sub> and sulfur dioxide (SO<sub>2</sub>) are represented in this modeling framework by a  $K_g$  value of 20 cm/sec, which leads to 20–30% uptake in the upper airways and the majority of the dose deposited in the lower conducting airways (bb region). This is consistent with modeling estimates of O<sub>3</sub> uptake in humans and animal inhalation experiments showing that the majority of O<sub>3</sub> uptake is in more distal regions of the respiratory tract (Miller et al., 1985; Overton, et al., 1987; Grotberg, et al., 1990; Sarangapani, et al., 2003; ICRP, 1994).

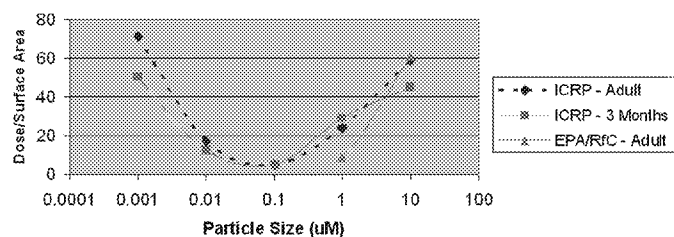
For non-reactive gases, the steady-state approach described in the RfC methodology was enhanced by the explicit inclusion of parameters representing hepatic blood flow and intrinsic clearance (Sarangapani, et al., 2003; RfC Methodology, Appendix I) to describe blood levels of parent compound and hepatic levels of metabolite. The steady state model was run with parameters for male adults and 3-month old infants, with

additional runs conducted for 1-year olds. Details of the methods and equations used for these simulations are described elsewhere (Ginsberg, et al., 2005).

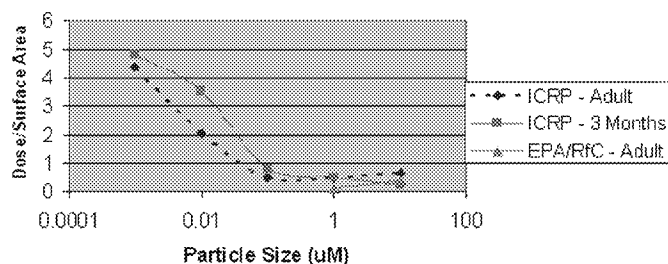
The key physiological parameter governing deposition of particles and gases in the RfC and ICRP default models is ventilation rate. If the chosen dose metric is the deposited fraction normalized to surface area, as proposed in the RfC methods, then regional surface area is also an important normalizing parameter in the calculation. The characteristics of the inhaled material, in particular particle diameter and its distribution, are key determinants of deposition as well. Parameter values for 3 month old children and adults are presented in Table 3. The largest difference in ventilation rate per surface area across age groups is in the PU region as neonates have relatively few alveoli at birth, leading to a large ratio of air flow per surface area in the 3 month old deep lung. The opposite situation exists in the bronchioles (bb region) in that its branched airway structure is believed to be nearly complete at birth leading to a relatively large surface area even at young ages.

#### DEFAULT MODEL RESULTS FOR YOUNG CHILDREN

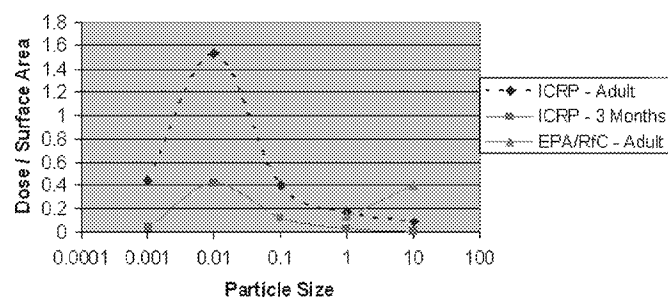
Particle deposition modeling results for the major respiratory regions are presented as average dose normalized to regional surface area ( $\mu\text{g}$  deposited/ $\text{cm}^2/\text{min}$ ) in Figures 4 to 7. Deposition estimates for the ET region show agreement between the RfC and ICRP models in the larger particle size range ( $> 1 \mu\text{m}$ ); however, comparisons across models are not possible below that range. Comparison of predictions for the 3-month old infant to male adult using the ICRP model did not reveal substantial differences in deposition in the ET region. Figure 5 shows a generally similar pattern predicted in the upper TB region with results for the 2 models and for the 2 age groups converging in the 1–10  $\mu\text{m}$  size range. Ultra fine particles having a diameter below 0.1  $\mu\text{m}$  are predicted to have nearly 2-fold higher dose in a 3-month old infant than in male adults. This profile reverses in the bronchiolar region as adult dosimetry is greater than in the 3-month old for ultra-fine particles (Figure 6). This prediction may be in part due to the



**FIG. 4.** Particle deposition predicted by various models in the ET region of 3-mo-old infants and male adults. Dose per surface area units in  $\mu\text{g}/\text{cm}^2/\text{min}$  at an inspired concentration of 1  $\mu\text{g}/\text{ml}$ . Simulations used ICRP (1994) or U.S. EPA RfC methodology (1994) models. Adapted from Ginsberg et al. (2005).



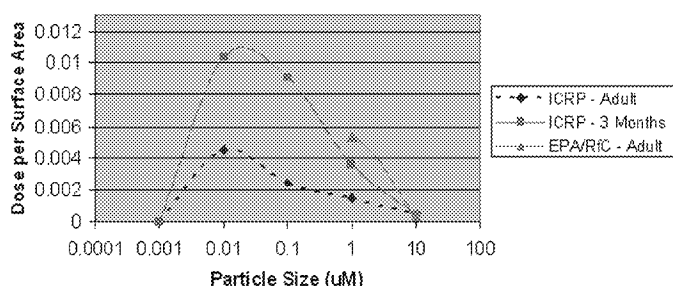
**FIG. 5.** Particle deposition predicted by various models in the upper trachea/bronchii (bb) region of 3-mo-old infants and male adult. Dose per surface area units in  $\mu\text{g}/\text{cm}^2/\text{min}$  at an inspired concentration of 1  $\mu\text{g}/\text{ml}$ . Simulations used ICRP (1994) or U.S. EPA RfC methodology (1994) models. Adapted from Ginsberg et al. (2005).



**FIG. 6.** Particle deposition predicted by various models in the bb (bronchiolar) region of 3-mo-old infants and male adults. Dose per surface area units in  $\mu\text{g}/\text{cm}^2/\text{min}$  at an inspired concentration of 1  $\mu\text{g}/\text{ml}$ ; particle size in micrometers. Simulations used ICRP (1994) or U.S. EPA RfC methodology (1994) models. Adapted from Ginsberg et al. (2005).

scrubbing out of ultra-fine particles in more proximal airways, which appears to be greater in children, and in part to the high surface area already developed in 3-month old bronchioles. Deposition predictions for the PU region shows greater deposition in a 3 month old infant compared to male adults for ultra-fine particles (Figure 7). The differential is approximately 2–4 fold. As with the other regions, model output for the 1–10  $\mu\text{m}$  range shows little across age or across model difference. Thus, if one were modeling particles in this size class only, the default RfC algorithm would yield results for adults that would also be reasonable estimates for young children. However, to address smaller particles, other models such as the ICRP or MPPD models would need to be used.

Results from using the default gas dosimetry algorithm across the spectrum of  $K_g$  values and for various regions of the respiratory tract are shown in Figure 8. It should be noted that for these calculations, the parameter value for  $K_g$  was assumed to be the same along the entire respiratory tract, and also to be the same in adults and children, despite differences that would occur due to differences in architecture noted above if the  $K_g$  were actually measured. As anticipated for Categories 1 and 2

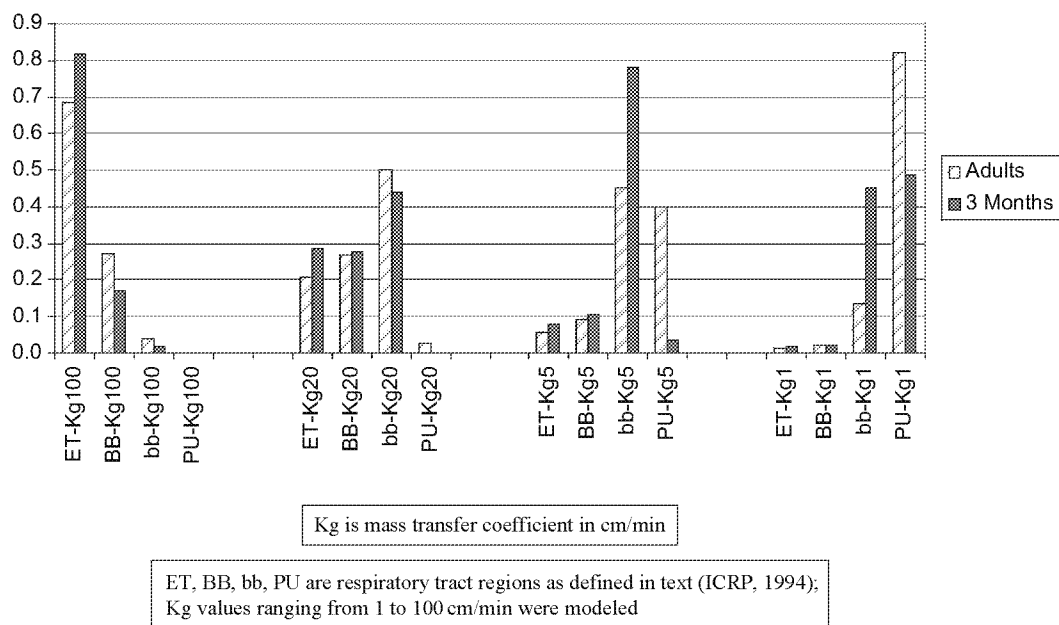


**FIG. 7.** Particle mass deposition predicted by various models in the PU region of 3-mo-old infants and male adults. Dose per surface area units in  $\mu\text{g}/\text{cm}^2/\text{min}$  at an inspired concentration of  $1 \mu\text{g}/\text{ml}$ . Simulations used ICRP (1994) or U.S. EPA RfC methodology (1994) models. Adapted from Ginsberg et al. (2005).

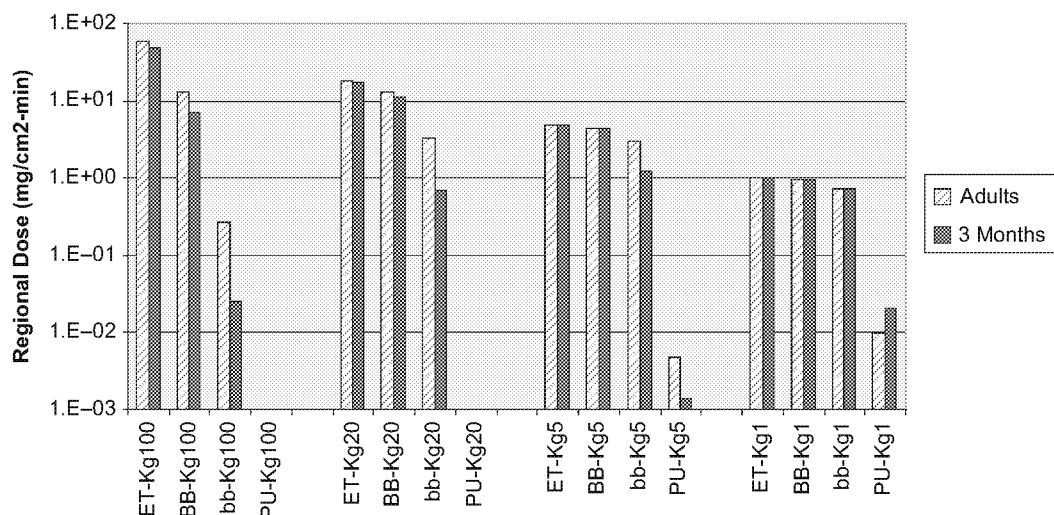
gases, these calculations predict extraction of high  $K_g$  gases occurring mainly in the ET region, with greater penetration of gases of intermediate reactivity (Category 2). As  $K_g$  decreases further to the low end of the range simulated, a substantial portion reaches the PU region. At this low reactivity it is likely that much of the deposited dose would be systemically absorbed rather than react locally. The modeling of the systemic absorption of gases with low reactivity (Category 3) is described below.

For interspecies extrapolation of respiratory tract effects, gas uptake is typically normalized to surface area to calculate

the default regional gas dose (RGD) used in a ratio (relative to the human RGD) as the interspecies dosimetric adjustment factor (DAF) in the RfC methods. Figure 9 shows estimates of the RGD for 3 month old children and adults, across the same range of  $K_g$  values as shown in Figure 3–6, expressed as  $\text{mg}/\text{cm}^2/\text{min}$  for a  $1 \text{ mg}/\text{ml}$  inspired concentration. Overall, the simulations show estimated RGD values that are greatest in the ET region where there is the highest rate of gas delivery per surface area. This is especially pronounced for high reactivity/solubility gases for which there is little penetration beyond the initial airway regions. The RGD generally declines with increasing distance in the respiratory tract due to less gas delivery to these regions. The estimated RGD values for 3 month old children are not markedly different from adult values. The increased % gas extraction in the bb region of 3 month old children (Figure 8) is counterbalanced by the lower rate of gas delivery to this region in this age group. This leads to RGD values that are somewhat lower than adult levels. At the lowest reactivity assessed ( $K_g = 1 \text{ cm}/\text{min}$ ) the dose to the PU region becomes greater in 3-month old infants than male adults but the reverse is true for  $K_g = 5 \text{ cm}/\text{min}$ , and in any case, RGD values in the PU region are still far below those in more proximal regions. These default modeling calculations suggest only minor differences in predicted respiratory dosimetry of reactive gases when comparing between 3-month old infants and male adults, but may largely reflect the assumption that the  $K_g$  was the same across the age groups.



**FIG. 8.** Percent extraction of reactive gases predicted by RfC methodology (U.S. EPA, 1994) in the respiratory tract of 3-mo-old infants and male adults. From Ginsberg et al. (2005).

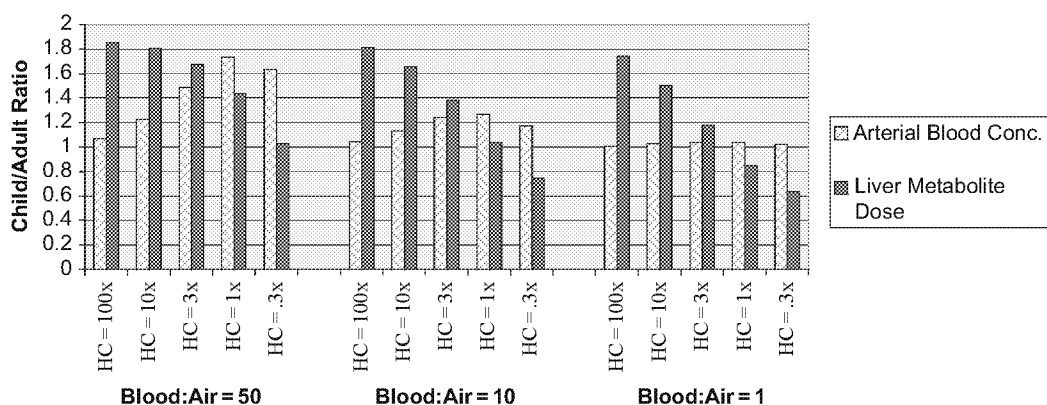


**FIG. 9.** Reactive gas dosimetry predicted for respiratory regions of 3-mo-old infants and male adults by the default model. ET, BB, bb, PU are respiratory tract regions as defined in text (ICRP, 1994);  $K_g$  is mass transfer coefficient in cm/min.  $K_g$  values ranging from 1 to 100 cm/min were modeled. From Ginsberg et al. (2005).

Rather than simulating specific Category 3 gases, Ginsberg et al. (2005) constructed a matrix of gases having a range of blood: air partition coefficients and intrinsic hepatic clearance. All gases were assumed to be substrates for CYP2E1, the hepatic CYP most commonly involved in metabolizing chlorinated solvents and simple aliphatic molecules (Guengerich, et al., 1991). Therefore, the developmental profile for this CYP was used to adjust intrinsic clearance for children at 3 months (30% adult function, Vieira, et al. 1996; Alcorn and McNamara, 2002). Figure 10 shows that gases which are readily extracted by the liver yield nearly a 2 fold more liver metabolite dose in 3-month old infant than adults. This is related to the fact that at such high extraction rates, hepatic

blood flow is the rate limiting step to metabolic clearance, with this limitation preventing the expression of the metabolic immaturity in CYP2E1 at this age. In contrast, parent compound blood concentrations are greater in children than in adults for low extraction chemicals since this is where the immaturity in CYP2E1 makes the biggest dosimetry difference. This pattern is accentuated in other simulations involving clearance by a CYP that is slower to mature (e.g., CYP1A2; Sonnier and Cresteil, 1998) (data not shown).

The ventilation rates for young children selected in these model exercises are low relative to those estimated by an alternative method for deriving inhalation rates recently published and presented at this workshop (Foos, et al., 2007; U.S. EPA,



**FIG. 10.** Comparison of infant–adult internal dosimetry predicted for a range of Category 3 gases using the steady-state default model as described in U.S. EPA (1994) and Sarangapani et al. (2003). HC signifies hepatic clearance relative to blood flow; blood:air partition coefficients ranging from 1 to 50 were modeled. From Ginsberg et al. (2005).

2006). The newer estimates are based upon basal metabolic rate and energy expenditure at different levels of activity represented in the Comprehensive Human Exposure Database (CHAD) described in a companion paper (Foos, et al., 2007). These inputs are then used to calculate oxygen ( $O_2$ ) demand and the ventilation rate needed to support this  $O_2$  demand. Use of these updated estimates would generally lead to greater child-adult differences in deposition in the exercises performed herein because of the sensitivity of the model to the ventilation rate input parameter.

## CONCLUSIONS FROM USE OF DEFAULT MODELS

Default models and algorithms were used in the assessment of risk from inhaled chemical and PM exposures for more than a decade. While they represent simplistic descriptions of gas uptake and particle deposition based largely on empirical measurements, they provide a useful default or screening level analysis of cross-chemical and cross-species differences in respiratory dosimetry. As shown above, they also were adapted to provide comparisons across age groups and thus inform risk assessments of inhaled toxicants in children. The simulations and calculations in this section suggest that 3-month old infants may receive a greater delivered dose of inhaled PM, especially for ultra-fine particles in the PU region. While reactive gas dosimetry did not show marked child-adult differences, this might change if the estimates of child ventilation rates derived from the alternative method discussed above are validated for use in dosimetry modeling. Internal dosimetry of Category 3 gases may be greater in children than adults with the difference (1) depending upon the chemical's blood:air partition coefficient, (2) rate of hepatic metabolism, and (3) whether the parent compound or metabolite is of most concern. Estimates of children's dosimetry may be improved with the development of more refined modeling as described in subsequent sections of this paper, and these improvements need to be incorporated into generally applicable models that are suitable for risk assessment.

## MULTI-PATHWAY MODELING OF PARTICLE DOSIMETRY IN THE RESPIRATORY TRACT OF CHILDREN

Chronic exposure to airborne PM is associated with increased morbidity and mortality, particularly in sensitive populations such as the elderly and children (Berglund et al., 1999; Beyer et al., 1998; Conceição et al., 2001; Gauderman et al., 2002). Most efforts to characterize the fate of inhaled particles in the lung have focused on adults, and few studies are available in children. Children present a special challenge because the growth and development of the airways are not fully characterized especially with respect to the degree that these vary at a particular age. Given that lung physiology and geometry differ markedly between children and adults

(Overton and Graham, 1989), it is important to explore dosimetry models that predict the deposition and clearance of PM in the extrathoracic and lower respiratory tract (LRT) airways of children.

## EXTRATHORACIC (ET) PARTICLE DEPOSITION: MEASUREMENTS AND MODELING IN CHILDREN

Morphometric studies of the ET airways were documented from cadaver studies in children (Bosma, 1986). However, the anatomical dimensions produced from this work are believed to overestimate nasal airway dimensions *in vivo* due to congestion, dehydration, and fixation artifacts (Swift, 1991). Previous studies in adults using magnetic resonance imaging (MRI) showed that the average cross-sectional area of airways *in vivo* was 33–50% (Guilmette et al., 1989) of that described in cadaver studies (Montgomery et al., 1979). These differences make MRI or CT data preferable when available for children and adults such as reported in hollow airway models (Swift 1991; Swift et al. 1994; Janssens et al. 2001). In these projects, a 4-week old infant (Swift et al., 1994), a 6-week-old infant (Swift, 1991) and a 9-month-old child (Janssens et al., 2001) were included. Particle deposition studies in these hollow airway models were conducted for a variety of particle sizes using spherical aerosol particles. However, the model of Janssens et al. (2001) is the only one that includes a larynx, which is a key anatomical feature for deposition of inhaled PM.

A number of models are used to predict particle deposition in the ET airways of young children and adolescents. Xu and Yu (1986) and Robinson and Yu (2001) simply use adult ET airway values and do not scale for children and adolescents. The ICRP (1994) models scale the ET airways as a function of tracheal diameter, as suggested by Yu and Xu (1987). The NCRP (1997) model uses an empirical equation derived from adult clinical data and assumes that children and adults possess the identical deposition efficiency at equivalent physical exertion levels. Recently, Cheng (2003) proposed empirical equations for particle losses due to diffusion and impaction in nasal and oral airways. These equations are based on clinical measurements in adults and *in vitro* measurements in hollow nasal and oral models.

Direct use or rescaling of the adult models based on tracheal diameter may lead to deposition overprediction and underprediction in the ET and lung respectively, in children (Asgharian, et al., 2004). While limited, it is best to use existing measurements to construct semi-empirical models of particle deposition in the ET region of children. For example, deposition measurements of 1– to 2–  $\mu$ m particles in the nasal airways of children in two age groups above and below 11 years are reported by Becquemin et al. (1991). Asgharian et al. (2004) developed models of ET deposition by impaction by fitting the above dataset to a functional relationship between particle deposition efficiency and impaction parameter. Similar

relationships are desirable for nano-sized and ultrafine particles in nasal airways and for all size particles in oral airways. At this time these remain important data gaps.

#### LOWER RESPIRATORY TRACT GEOMETRY: MEASUREMENTS AND MODELS IN CHILDREN

Information on the lung geometry of children for use in particle deposition models is also extremely limited. A child's lung is not a scaled-down version of an adult lung, so that direct measurements are requisite for accurate descriptions of particle deposition in various age groups. It is generally agreed that the number of bronchial airways are complete at birth (Reid, 1984), but the rate of growth of proximal and distal airways changes with age. The alveolar region consists of respiratory bronchioles, ducts, and a terminal cluster of alveolar sacs at birth (Charnock and Doershuk, 1973). The number and size of alveoli increase with age, with the number stabilizing before the age of 8 (Dunnill, 1962; Reid, 1984).

Despite these data limitations, several models of bronchial airway growth in children were proposed (Xu and Yu, 1986) using some simplified scaling approaches. These models assume the same growth rate for all airways, the same as those of main bronchi. Phalen et al. (1985) proposed a different model of airway growth from measurements of the right upper lobe of 20 casts of children's lungs. They found a relationship between airway size and subject body length per airway generation. The children's geometries used in the ICRP (1994) and NCRP (1997) dosimetry models are based on the data of Phalen et al. (1985). Thus, these geometry models may not be true representations of lung geometry in the sense that they are based on a limited dataset.

Additional information on children's lung airway parameters is available. Mortensen et al. (1983) at the Utah Biomedical Test Laboratory (UBTL) made complete measurements of tracheobronchial (TB) airway lengths, diameters, and branch angles for the first 10 generations of the lungs of 11 children between 3 months and 21 years old. Ménache et al. (personal communication) developed single-path, whole-lung and lobar lung models for children based on this dataset and information on distal airway dimensions published in the peer-reviewed literature (Weibel, 1963). First, the number of conducting airway generations was estimated from available information in the literature (Weibel, 1963; Yeh and Schum, 1980; Horsfield and Cumming, 1968; Beech et al., 2000). An average of 16 generations was used for the conducting airways in the typical-path model. This number for the lobar model consisted of 15 generations in the right upper, right middle, and left upper lobes, and 16 and 17 generations in the left and right lower lobes respectively. In addition, three generations of respiratory bronchioles and 4 generations of alveolar ducts were selected (Pinkerton et al., 2000; Weibel, 1963; Yeh and Schum, 1980; Horsfield and Cumming, 1968; Haefeli-Bleuer and Weibel, 1988; Hislop and Reid, 1974; Reid, 1984).

Ménache et al. (personal communication; Asgharian, et al., 2004) then determined airway dimensions for the conducting and respiratory airways. Measurements from Mortensen et al. (1983) were used for the first 10 generations of the lobar models. The airway dimensions of the typical model in the first 10 generations were simply averaged length and diameter values per generation. For the remaining airway generations, Ménache et al. (personal communication) used an equation proposed by Weibel (1963) to estimate the missing dimensions in each generation. The equation proposed by Weibel (1963) related airway dimensions (e.g., length and diameter) to generation number. The coefficients of the equation were obtained by fitting the equation to the airway dimensions of the first 10 generations plus published airway dimensions of the terminal bronchioles, respiratory bronchioles, alveolar ducts, and alveolar sacs.

Finally, Ménache et al. (personal communication; Asgharian, et al., 2004) used available information to estimate the following for the respiratory airways at different age groups: number (Weibel, 1963; Dunnill, 1982; Thurlbeck, 1988), diameter (Hislop et al., 1986; Dunnill, 1962; Weibel, 1963), and distribution of alveolar volume (Weibel, 1963; Haefeli-Bleuer and Weibel, 1988). This resulted in typical-path and 5-lobe symmetric but structurally different lung geometries for 11 age groups ranging from 3 months to 21 years. The completed airway model was uniformly scaled to predicted functional residual capacity. The expressions derived by Overton and Graham (1989) were used to estimate lung and breathing parameters at each age group.

#### LOWER RESPIRATORY TRACT: PARTICLE DEPOSITION MODELING IN CHILDREN

A variety of mathematical models were developed to predict particle deposition in the respiratory tract. The range of models extends from empirical models that do not incorporate lung geometry explicitly (Rudolf et al., 1986, 1990; ICRP, 1994), to typical-path models (Yu, 1978) based on symmetric lung geometry, and mathematically more complex, multiple-path models that are based on asymmetric lung structure (Anjilvel and Asgharian, 1995; Asgharian and Anjilvel, 1998; Asgharian et al., 2001). In addition, there are stochastic lung deposition models that sequentially generate one airway at a time along a given lung pathway and calculate particle deposition for that airway. By repeating deposition calculations along many randomly-generated pathways, an average deposition fraction is calculated statistically (Koblinger and Hofmann, 1985, 1990). While empirical models are accurate and easy to use, they can not be used to extrapolate outside the range of measured data (e.g., the range of particles sizes used to measure deposition efficiency). Typical-path lung models are useful for obtaining average regional and overall deposition of particles in the lung but do not provide information regarding distribution of deposited particles in the lung. More detailed

and site-specific deposition information are gained by using deterministic and stochastic multiple-path deposition models in which more accurate assessment of the lung structure is included.

The multiple-path particle dosimetry (MPPD) model calculates deposition fractions of inhaled particles in all airways of the respiratory tract during a single breathing cycle (CIIT Centers for Health Research, 2004). In general, the process of deposition modeling in the MPPD model consists of 4 steps. First, lung ventilation is calculated to determine how the inhaled particles are distributed throughout the lung. For a uniformly expanding and contracting compliant lung, airflow rate at any location in the lung is proportional to the volume distal to that location. Second, the combined deposition efficiency of particles in each airway by various deposition mechanisms is calculated. Deposition efficiency is the fraction of traveling particles through an airway that are deposited as a result of external forces exerted on them. Third, particle transport in the lung is simulated by solving the transport equation shown below to yield particle penetration and deposition. The transport equation in a uniformly expanding and contracting lung is described by the following expression:

$$\frac{\partial}{\partial t}(Ac) + \frac{\partial}{\partial x}(Qc) = -\lambda c \quad (1)$$

where  $A$  is the airway cross-sectional area,  $c$  is particle concentration,  $Q$  is the airflow rate,  $\gamma$  is related to particle deposition efficiency, and  $x$  and  $t$  are distance along the airway and elapsed time respectively. Equation (1) is solved by method of characteristics to find particle concentration at the exit of the airway.

The last step in deposition modeling involves performing a mass balance on the particles in the lung to calculate fraction of inhaled particles that are deposited per airway, generation, region, and lobe of the lung. Assuming a steady-state, steady-flow process, the mass balance on the traveling particles in an airway during a single breathing cycle (inhalation, pause, and exhalation) written in the following form:

$$m_{dep} = m_{ini} + m_{in} - m_{out} - m_{rem} \quad (2)$$

where  $m_{dep}$  is the mass deposited in an airway,  $m_{ini}$  is the mass initially in the airway,  $m_{in}$  and  $m_{out}$  are the masses of particles that enter and leave the airway at the end of the breath, respectively, and  $m_{rem}$  is the undeposited mass in the airway.

The MPPD model for deposition in children is practically identical to the MPPD model for adults except for the differences in lung geometry and lung and breathing parameters, which may be due to assumptions regarding scaling. Earlier deposition models for children are based on simplified

typical-path lung structures that were obtained from limited airway measurements in children's lungs (Phalen et al., 1985) and are useful for regional prediction of deposition. The availability of lobar lung geometry (Ménache et al., personal communication) paved the way for more advanced deposition modeling in the lungs of children. Asgharian et al. (2004) used the lung geometries from Ménache et al. (personal communication) and developed a lobar deposition model for children based on the multiple-path analogy described above. The MPPD model is used below to compare deposition between children and adults (Asgharian et al., 2004; CIIT Centers for Health Research, 2004).

## RESULTS OF THE MULTIPLE-PATHWAY PARTICLE DOSIMETRY (MPPD) MODEL IN CHILDREN

Measurements of particle deposition fraction in the thoracic airways of children via oral breathing was reported by Becquemin et al (1987;1991), Bennett and Zeman (1998), and Schiller-Scotland et al. (1994). These datasets, which provide information for ages 3 months to 21 years, were used for verification of the MPPD model. Figure 11 is the plot of MPPD model predictions against these measurements. Each data point in the figure indicates the predicted deposition fraction against the measured values in a different subject. The age, lung size, and lung breathing parameters were different for each subject. Particle size characteristics were also different in each experiment and were used accordingly to obtain model predictions. In addition, plotted in the figure is the identity line, the line which corresponds to a perfect match between MPPD model predictions and experimental data. The closer the points in Figure 11 to the identity line, the better the agreement between

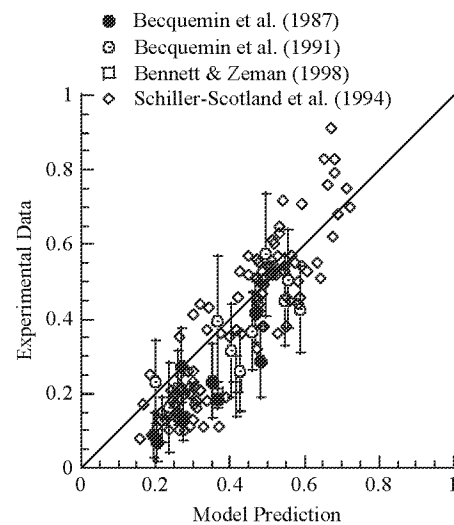


FIG. 11. Comparison of predicted total lung deposition fraction with experimental measurements.

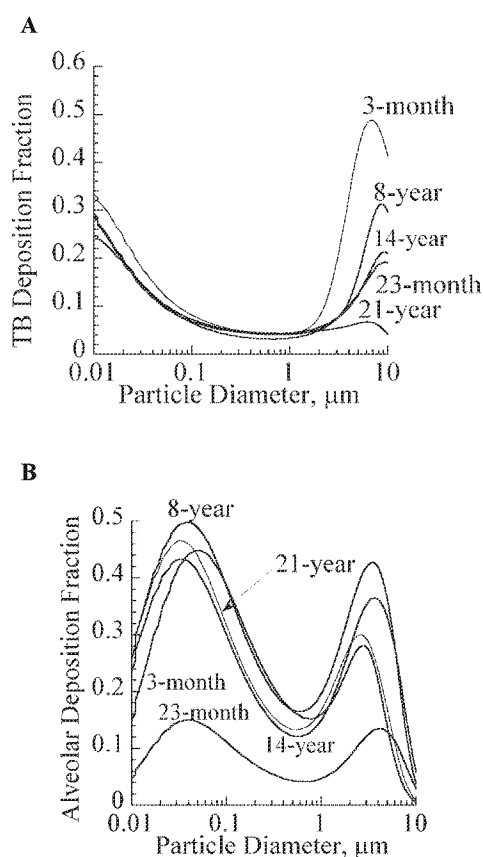
predictions and measurements. Overall, a good comparison between measurements and predictions of lung deposition fraction were observed. However, there was a slight over-prediction of deposition fraction.

Particle deposition models for various ages were exercised across a wide range of particle sizes, with results expressed as % deposition or as deposited dose based upon normalization to lung volume. Predicted particle deposition fractions in the TB region via nasal breathing were almost the same for particles from 0.01 to 2  $\mu\text{m}$  except for 3-month-old infants, where deposition fractions were noticeably higher for particles smaller than 1 (Figure 12A). Due to increased nasal deposition with age (Asgharian et al., 2004), TB deposition was significantly higher in children for fine and coarse PM. Deposition fraction of particles in the alveolar region for all ages showed one peak for particles smaller than 0.1  $\mu\text{m}$  and a second peak for particles larger than 1  $\mu\text{m}$  (Figure 12B). This alveolar deposition pattern appears to be due to the effects of particle filtering in the TB region. Despite having similar shapes, the deposition curves intersected one another, indicating that there was not a

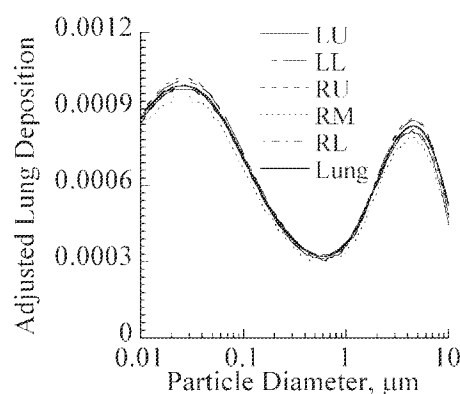
clear pattern for deposition fraction with age at a given particle size for these airway geometry models.

Inspection of various parameters controlling particle deposition revealed that differences in lobar deposition depended primarily on lobar volume such that the right middle lobe, which has the least lobar volume, yielded the smallest deposition fraction and lower lobes with greatest volumes were predicted to have the largest deposition. In fact, when adjusted lobar deposition fraction (defined as deposition fraction in a lobe divided by its corresponding lobar volume) was calculated for different particle sizes in all 5 lobes, almost a single functional relationship was observed, indicating similar relative doses among the lobes (Figure 13). In addition, the line representing the ratio of lung deposition fraction to lung volume yielded a similar outcome.

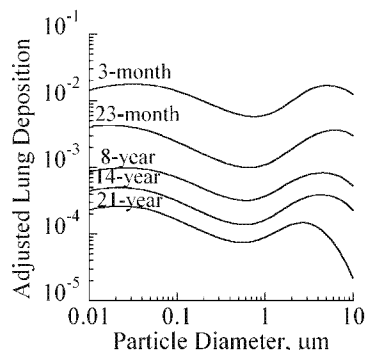
Adjusted lung deposition fraction is a unique feature of the lung for a particular age and thus presents a means for comparing lung dose across age groups. Figure 13 is calculated for an 8-year old but could be estimated for any age group: deposition fraction for different regions of the lung is the same for a given particle size when normalized with the respective volume of the region. Thus, a single deposition curve such as that shown in Figure 13 exists at each age group. This unique feature of the adjusted lung deposition fraction presents a means for comparing lung dose across age groups. Using the MPPD model, Ménache et al. (personal communication; Asgharian, et al., 2004) calculated deposition fraction per lung volume for different ages and particle diameters between 0.01 and 10  $\mu\text{m}$ . In this modeling framework, a clear pattern with age emerged (Figure 14). At a given particle size, the adjusted lung deposition fraction was highest in infants and decreased with age. The differences in adjusted deposition across ages were related to the differences in lung volume. Due to faster lung growth at



**FIG. 12.** Age-related mass deposition fraction via nasal breathing predicted as a function of particle size in TB (A) and alveolar (PU) (B) regions by the MPPD model.



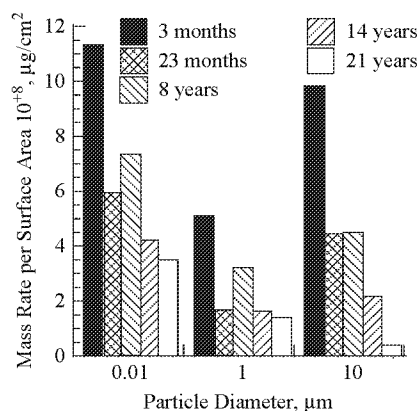
**FIG. 13.** Adjusted mass deposition fraction of particles predicted by the MPPD model for the entire lung and each lobe of an 8-yr-old child. Deposition fraction values are unitless, adjusted for lung region volume.



**FIG. 14.** Adjusted mass deposition fraction in the lungs of children and adults predicted by the MPPD model. Deposition fraction values are unitless, adjusted for lung region volume.

early stages of life, the difference in adjusted deposition was greater in younger ages. The change in adjusted deposition was more gradual after the age of 8 years.

Lung injury is related to local rather than regional deposition. Deposition per surface area is an indicator of local dose. The data in Figure 14 are transformed to be expressed as mass deposition rate per lung surface area in Figure 15. This method of normalization yields a similar trend as the dose/lung volume results, with 3 month-old children having up to an order of magnitude greater deposition dose than adults. This trend occurs at all particle sizes modeled. In each age group, deposited mass per lung surface area is greatest for ultrafine particles, decreases for fine (1  $\mu\text{m}$ ) particles and then increases again for coarse particles. Regarding cross-model comparisons, MPPD and ICRP models predict similar regional deposition fractions of various sized particles in adults (Ashgarian, et al., 2001).



**FIG. 15.** Particle deposition dose of mass per lung surface area at various ages.

## COMMENTS ON PARTICLE CLEARANCE MODELING

Internal doses may be accurately described by particle deposition alone if the particles exert their primary action on the epithelial surface (Dahl et al., 1991). For longer term effects, however, the deposited dose may not be as appropriate because particles clear at various rates from different lung compartments so that some dose is retained (Jarabek et al., 2005). To characterize chronic effects of inhaled particles, models need to calculate retained dose within the respiratory tract by accounting for clearance pathways (U.S. EPA, 1994; 1996; 2004). Recent workshops regarding risk assessment approaches to fiber and particle toxicity recommended that species-specific toxicokinetic models need to be used to predict particle clearance and retention in the lungs (ILSI, 2000; Greim et al, 2001).

Current data gaps necessitate the calculation of deposition and clearance separately in default algorithms, but the two processes are coupled and improved models provide for integrated calculation of retention as the net result of deposition minus clearance (ICRP, 1994; NCRP, 1997). In order to solve for deposition, available mechanistic models idealize the particle transport process by mathematically converting the spread of particles as one-dimensional penetration in the lung and 3-dimensional deposition in airways by various loss mechanisms. In addition, the lung geometry is selected as a dichotomous, symmetric or asymmetric network of cylindrically-shaped airways. Consequently, dose predictions are reliable at the regional level for insoluble PM.

The ICRP respiratory tract dosimetry model accounts for clearance from the respiratory tract as a result of dissolution of particles or elution of their constituents, followed by absorption of the dissolved constituents into cells proximate to the particles, or into the circulatory system for redistribution or excretion. Current efforts by the ICRP are underway to compile these critical data for radiological particles (Bailey et al., 2003). Previous simulation exercises using realistic dissolution-absorption half-times for different PM diameter size modes (fine, intermodal, and coarse) used in support of the NAAQS for PM showed that particle solubility rates are dominant determinants of retained lung burdens of inhaled particles of ambient aerosols (U.S. EPA, 1996; Snipes et al., 1997).

Various assumptions similar to those made for modeling mucociliary clearance in adults are made to estimate the retention of insoluble particle in the conducting airways of the child lung. For example, mucus layer travels at a constant velocity with an effective thickness that is small compared to lumen diameter and mucus production rates are the same in all terminal bronchioles. Since tracheobronchial structure of the lung is complete at birth, modeling of clearance in this region in children generally follows that of adults. Because children have smaller lung geometry, particles have a shorter distance to travel to be cleared and so may be cleared faster than in adults for the same mucous velocity (Figure 16). However, mucous

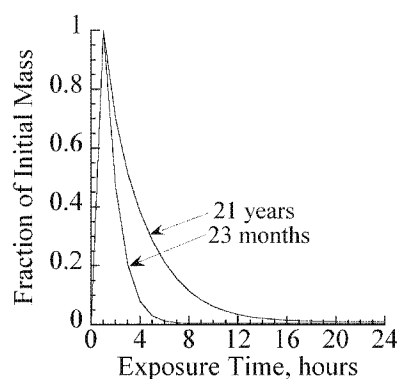


FIG. 16. Simulated fraction of retained mass (unitless) in the TB region of children after a 1-h exposure to 1- $\mu$ m particles.

velocity is not necessarily constant across age. Wolff (1992) reported the tracheal mucus velocities in beagle dogs at different ages. The data showed that the mucus velocity increased to a maximum value in young adults and then declined with age. He argued that age-related changes in canine lung functions are most similar to those in humans. Thus, mucus velocity in humans may be expected to increase after birth, reach peak values at puberty, and then slowly decrease with age. Clearly, additional detailed information regarding the change in mucus velocity with age is required to realistically estimate the retained dose in the lungs of children.

The MPPD and ICRP models approach clearance in the pulmonary region in an analogous fashion (Jarabek et al., 2005). The clearance model is comprised of fast, medium, and slow compartments to describe the following: macrophage phagocytosis, physical particle translocation on mucociliary escalator, and clearance to the lymph nodes via the alveolar interstitium. The clearance rates used for the fast ( $\gamma_{fast}$ ), medium ( $\gamma_{med}$ ) and slow ( $\gamma_{slow}$ ) compartments were 0.02, 0.001 and 0.0001 /day, respectively. The slow compartment also clears via lymphatic channels ( $\gamma_L$ ) at a rate of 0.00002 /day. While the entire pulmonary region is considered as one compartment in the ICRP model, a distinct alveolar compartment distal to each terminal bronchial was assumed in the MPPD model. Accordingly, particle removal from each alveolar (acinar) region was calculated independently. Deposition is then apportioned to the fast (30%), medium (60%), and slow (10%) clearance compartments. This leads to a more accurate characterization of clearance than combining all the alveolar zones into one and then computing clearance for a single alveolar region (Jarabek et al., 2005). Simulation exercises using this model predict a qualitatively similar pattern of retained dose as that of deposited dose in the TB region (Jarabek et al., 2005).

## CONCLUSIONS FOR MULTI-PATHWAY PARTICLE DOSIMETRY MODELING

Recent improvements in dosimetry modeling enabled more accurate prediction of deposition of particles in the lungs of adults and children. The models were validated by comparing predicted versus measured deposition in the entire lung of children at different age groups. However, additional morphometric measurements of airway parameters, particularly in the deep lung, are needed to refine these modeling estimates. Further needed are deposition measurements in ET and TB airways across a spectrum of children's ages.

Many childhood lung disorders such as asthma occur in the TB region (Bierbaum and Heinzmann, 2007). Particle deposition and clearance in this region may be crucial to the onset or exacerbation of lung diseases. The current results show that children have the potential for greater total lung mass deposition of a wide range of inhaled PM, but these results need better specification in terms of sub-regions where this across age deposition discrepancy may be greatest. More refined models that are built upon expanded measurements in children are needed to fully understand the implications of this research. Further, particle clearance rates should be included in children's dosimetry models, because of its significant impact on predicted doses. The limitations in mucus-based clearance data are thus another important data gap.

Thus, based on dosimetric considerations, children may face an increased health risk from exposure to airborne PM compared with adults. The risk becomes potentially greater if children are also more sensitive to a given dose of particles due to incomplete development of body defense or repair mechanisms. Improved children's morphologic, deposition and clearance data are needed to improve upon current models.

The particle models presented herein are based on poorly soluble particles. Key physicochemical input parameters are particle diameter and distribution. The exercises presented herein were performed using monodisperse aerosols, but previous simulation exercises demonstrated that consideration of polydisperse exercises influence the predicted deposition and retained dose estimates by at least 2 to 3-fold (U.S. EPA, 1996; Snipes et al., 1997; Jarabek et al., 2005). Evaluation of the influence of polydispersity on predictions for children need to be performed. Other major determinants of deposition and retention such as hygroscopicity (ICRP, 1994; Schroeter et al., 2001) and physical dissolution also need to be simulated in age-appropriate models. Additionally, recent evidence suggests when different types of particles are compared, inhaled dose may be more appropriately expressed as particle volume, particle surface area, or number of particles rather than mass, depending on the adverse effect being evaluated (Oberdörster et al., 1994). Different dose metrics vary both based on whether particle mass or number is used as the internal measure of dose, and on the normalizing factor (e.g., ventilatory units, alveolar units, or alveolar macrophages) (Snipes et al.,

1997; Jarabek et al., 1995). Thus, age-specific data to construct such dose metrics for children are needed.

### FACTORS INFLUENCING THE DOSIMETRY OF REACTIVE GASES IN THE LOWER RESPIRATORY TRACT OF CHILDREN

This section focuses on the distribution of reactive gases in the conducting airways (tracheobronchial region) of the LRT. While default models typically divide the airways into a few well-mixed regions and predict the overall extraction of reactive gas in each region, other models such as those used in the regulatory arena for  $O_3$  and formaldehyde describe mass transfer in a more anatomically-accurate lung structure (Miller, et al., 1985; Overton et al., 1987;2001; Weibel, 1963). This section extends the default models by considering the generation-by-generation distribution of reactive gases along a conducting airway path. Simulations are conducted for both adults and children by using age-appropriate breathing and anatomical parameters.

Once distributed by respired air flow among local sites within the lungs, a reactive gas must diffuse through the mucus blanket before it reaches the underlying epithelial cells (Figure 17). Mucus protects these cells by providing substrates that combine with a reactive gas to form benign products. On the other hand, some of the substrates present in mucus might combine with a reactive gas to form secondary toxic products. For example,  $O_3$  is a reactive gas that is detoxified by endogenous antioxidants such as uric and ascorbic acid, but also reacts with polyunsaturated fatty acids to form aldehydes that damage epithelial cell membranes (Bhalla, 1999).

The specific goal of this section was to examine the uptake distribution of gases of different chemical reactivity along the gas-mucus, as well as the mucus-tissue, interfaces in the tracheobronchial tree of children of various ages. The effect of different ventilation rates associated with different levels of physical exertion was also considered. To make the computations concrete, physical-chemical parameter values appropriate

for  $O_3$  were employed, including an environmentally-relevant inhaled concentration of 0.1 parts per million by volume (ppm).

### MATHEMATICAL MODEL FOR OZONE UPTAKE IN THE TRACHEOBRONCHIAL REGION

Reactive gas dosimetry was analyzed in a tracheobronchial tree consisting of 15 generations of symmetrically-branched airway bifurcations. This modeling exercise began with air entering the trachea and thus did not simulate reactions possible in the ET region. Each airway was represented by a convection-diffusion model of the gas-filled lumen and a diffusion-reaction model of the surrounding mucus layer (Santiago et al., 2001). The two models were coupled at the air-mucous interface by an overall mass transfer coefficient ( $K_g$ ) that incorporated the diffusion resistances of both the mucus layer and an adjacent "unstirred" gas layer. The key assumptions were: a steady flow of inspired gas through the airway lumen; and a first-order chemical reaction of the gas within the mucous layer.

Numerical simulations of reactive gas uptake utilized airway parameters specific to children of different ages. The lengths and diameters of airway branches (Table 4) as well as lung ventilation rates (Table 5) were taken from Phalen and associates (1985). The individual mass transfer coefficients for reactive gas transport across the unstirred gas layer ( $k_g$ ) were computed from one of three established correlations for fully-developed flow through straight tubes (Treybal, 1980). The correlation used in a given situation depended on whether the gas flow was turbulent or laminar and, in the latter case, whether the concentration profile was developing or fully-developed.

It was also necessary to specify parameters within the mucus layer. The liquid-phase diffusion coefficient ( $D_m = 2.66 \times 10^{-5} \text{ cm}^2/\text{sec}$ ) and the thermodynamic gas-liquid partition coefficient for  $O_3$  ( $\lambda = 6.9$ ) were adopted from the work of Miller and coworkers (1985). The first-order reaction rate constant of  $O_3$  with mucus substrates ( $k_r$ ) was estimated as 1198 /s employing theoretical computations (Miller et al., 1985), 250,000/ sec from continuous exposure measurements in the nasal cavities (Santiago et al. 2001) and  $8 \times 10^6$  /sec from bolus exposure measurements in the lower airways (Bush et al., 2000). A midrange  $k_r$  of  $10^5$  /sec, corresponding to a moderate reaction rate, was employed in most of the analyses in this section. To estimate the mucus thickness ( $\delta$ ) in the airways of different aged children, it was first assumed that the mucus thickness in an adult's lungs declines in a linear fashion from 10  $\mu\text{m}$  in the trachea to 0.1  $\mu\text{m}$  in a 15<sup>th</sup> generation airway; this is similar to the scheme used by Miller and associates (1985). The mucus thickness in a particular generation of a child's lung was scaled down by the ratio of the child-to-adult airway diameters (Table 6). The individual mass transfer coefficients across the mucus layer ( $k_m$ ) were then computed using the results of the diffusion-reaction model shown schematically in Figure 18.

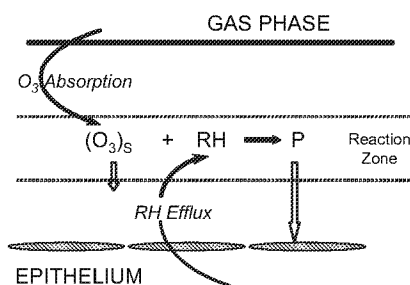


FIG. 17. Diffusion reaction of ozone within the mucous layer. RH=reactive substrate; P=reaction product (may or may not be toxic).

**TABLE 4**  
Airway Lengths (L) and Diameters (D) (mm)

Gen	Age (yr)											
	0		4		8		12		16		18	
	L	D	L	D	L	D	L	D	L	D	L	D
0	26.5	5.30	53.5	10.7	65.0	13.0	76.5	15.30	86.50	17.3	89.0	17.8
1	15.8	4.34	25.5	8.06	29.6	9.65	33.7	11.24	37.4	12.6	38.3	13.0
2	5.96	3.28	9.74	5.77	11.4	6.82	13.0	7.88	14.36	8.80	14.7	9.03
3	3.89	2.59	6.75	4.37	7.97	5.13	9.19	5.89	10.25	6.55	10.5	6.71
4	3.71	1.85	5.60	2.77	6.41	3.16	7.21	3.55	7.91	3.89	8.09	3.97
5	2.78	1.41	4.56	2.17	5.32	2.49	6.08	2.81	6.74	3.09	6.91	3.16
6	2.22	1.14	3.41	1.79	3.91	2.07	4.42	2.34	4.86	2.58	4.97	2.64
7	1.98	0.85	2.68	1.22	2.98	1.39	3.28	1.55	3.54	1.69	3.61	1.72
8	1.92	0.70	2.35	0.92	2.54	1.01	2.72	1.10	2.88	1.18	2.92	1.20
9	1.75	0.64	2.24	0.80	2.44	0.87	2.65	0.94	2.83	1.00	2.88	1.01
10	1.64	0.58	2.02	0.69	2.18	0.73	2.34	0.78	2.48	0.82	2.52	0.83
11	1.57	0.51	1.89	0.57	2.03	0.59	2.17	0.61	2.29	0.63	2.32	0.64
12	1.50	0.50	1.77	0.55	1.89	0.57	2.00	0.59	2.10	0.61	2.13	0.61
13	1.43	0.47	1.65	0.50	1.74	0.52	1.83	0.53	1.91	0.54	1.93	0.55
14	1.36	0.45	1.52	0.47	1.59	0.48	1.66	0.49	1.72	0.50	1.74	0.50
15	1.30	0.43	1.41	0.44	1.45	0.44	1.50	0.45	1.54	0.45	1.55	0.45

*Note.* Source: Computed from the equations of Phalen et al. (1985). Gen=airway generation of a symmetrically bifurcating tracheobronchial tree. Gen 0 is the trachea and Gen 15 corresponds to the terminal bronchioles.

**TABLE 5**  
Ventilation Rates Used in Reactive Gas Modeling

Age (yr)	Weight (kg)	Height (cm)	Minute volume (L)		
			Quiet breathing	Light exertion	Heavy exertion
0	3.3	50	1.52	3.00	8.92
4	16.4	104	3.18	6.34	19.00
8	27.0	127	4.53	9.05	27.10
12	43.0	150	6.56	13.10	39.30
16	63.0	170	9.10	18.20	54.60
18	70.0	175	10.0	20.0	60.0

*Note.* Source: Phalen et al. (1985).

The overall mass transfer coefficient for the unstirred gas and mucus layers was computed by combining the individual mass transfer coefficients (Treybal, 1980).

$$K_g = (1/k_g + 1/k_m)^{-1}$$

The final results for  $K_g$  during quiet breathing for a gas with a moderate reactivity of  $k_r=10^5$  /sec is shown in Table 7. in addition, shown in this table is % overall diffusion resistance

that is attributable to the mucus layer. Notice that for all ages and all airways, the controlling resistance for reactive gas uptake is in the mucus layer rather than in the unstirred layer of the respired air.

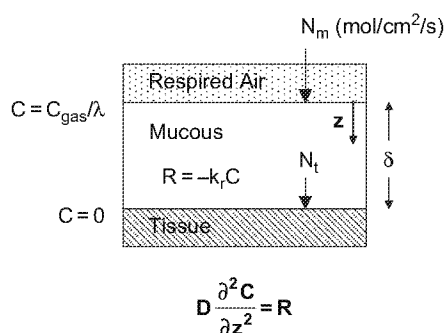
#### RESULTS FOR LRT MODEL OF OZONE UPTAKE

Figures 19 through 21 demonstrate how the flux of  $O_3$  (i.e., amount absorbed per unit time per unit surface area) varies from generation to generation along the gas-mucous interface

**TABLE 6**  
Mucous Thickness ( $\mu\text{m}$ ) Scaled From Adults by Relative  
Airway Diameter

Gen	Age (yr)					
	0	4	8	12	16	18
0	2.98	6.01	7.30	8.60	9.72	10.00
1	2.34	4.35	5.20	6.06	6.80	6.99
2	1.69	2.98	3.52	4.07	4.54	4.66
3	1.28	2.16	2.53	2.91	3.23	3.31
4	0.87	1.30	1.49	1.67	1.83	1.87
5	0.63	0.97	1.11	1.26	1.38	1.42
6	0.49	0.76	0.88	1.00	1.10	1.12
7	0.34	0.49	0.56	0.62	0.68	0.69
8	0.27	0.35	0.38	0.42	0.45	0.46
9	0.23	0.29	0.31	0.33	0.36	0.36
10	0.19	0.23	0.24	0.26	0.27	0.28
11	0.16	0.18	0.18	0.19	0.20	0.20
12	0.14	0.16	0.16	0.17	0.17	0.18
13	0.12	0.13	0.14	0.14	0.14	0.14
14	0.11	0.11	0.12	0.12	0.12	0.12
15	0.09	0.10	0.10	0.10	0.10	0.10

*Note.* Gen=airway generation of a symmetrically bifurcating tracheobronchial tree. Gen 0 is the trachea and Gen 15 corresponds to the terminal bronchioles. In the 18-yr-old lung, mucous thickness decreased in a linear fashion from 0.1  $\mu\text{m}$  in Gen 0 to 0.1 in Gen 15 (Miller et al., 1985). For younger lungs, the mucous thickness in a given Gen was scaled down by the ratio of the airway diameter in the young lung to the airway diameter in the same Gen of the 18-yr-old lung.



**FIG. 18.** Diffusion-reaction model of gas transport in the mucous layer. In this quasi-steady-state model, diffusion  $D(\partial^2 C / \partial z^2)$  is balanced by the first-order reaction rate  $R$  of reactive gas with endogenous substrates.  $N_m$  is the overall flux of reactive gas into the mucous layer, whereas  $N_t$  is the flux reaching the epithelial surface.

( $N_m$ ) and along the mucus-tissue interface ( $N_t$ ) when a constant concentration of 0.1 ppm is inhaled.

Figure 19 illustrates the sensitivity of  $N_m$  and  $N_t$  to the reactivity of the gas in mucous ( $k_r$ ). For highly reactive gases

(bottom panel),  $N_m$  is substantial in the proximal conducting airways where most of the gas is scrubbed out of the airstream, analogous to what happens in the ET region in models where this compartment is present. Flux into the tissue ( $N_t$ ) is negligible in all the conducting airways since the gas is so reactive in the mucus layer. For slowly reacting gases (top panel), the  $N_m$  and  $N_t$  distributions are quite similar, with a clear maximum in dose appearing at an intermediate airway generation. For moderately reacting gases (middle panel), the  $N_m$  distribution was similar to that observed for highly reactive gases, while the  $N_t$  distribution was similar to that observed for slowly reacting gases.

Figure 20, produced for a moderately reacting gas, illustrates the effect of different ventilation rates imposed by different levels of exertion. As exertion increases, the magnitudes of  $N_m$  and the peak values of  $N_t$  rise. Figure 21 illustrates the effect of age during quiet breathing. As age increases, the longitudinal distributions of both  $N_m$  and  $N_t$  shift distally toward the respiratory airspaces.

## DISCUSSION OF RESULTS FOR OZONE UPTAKE IN THE LRT

Assuming a moderate reaction rate, virtually all of the  $\text{O}_3$  inhaled by children 0–8 years old was absorbed in the mucus lining layer of the tracheobronchial tree during quiet breathing ( $N_t$  falls to 0 in the terminal airway generation). When gases have a lower reactivity or breathing occurs at higher ventilation rates, less gas is extracted by the mucus layer so that more gas penetrates to the respiratory airspaces. Increased penetration of  $\text{O}_3$  to the respiratory zone also occurs as an individual ages beyond 8 years. Because the overall mass transfer coefficient is not sensitive to age (Table 7), this must be attributed to the increase in the ventilation rate and decrease in the airway surface-to-volume ratio that occurs with aging.

Under all circumstances, increasing the assumed reactivity resulted in a higher  $\text{O}_3$  dose to the mucus surface but a lower  $\text{O}_3$  dose to the underlying tissue. If all the products of  $\text{O}_3$ -substrate reactions were non-toxic, this suggests that a rapidly-reacting gas might produce less tissue damage than a slowly-reacting gas. However, if the reaction products were toxic, then the entire flux of reactive gas across the mucus surface may result in tissue damage, and a rapidly-reacting gas might produce as much tissue damage as a slowly-reacting gas.

The shapes of the uptake distributions suggest that there are focal regions where tissue damage occurs. Longitudinal hot spots of  $N_t$  appeared in the distal portion of the conducting airways when low to moderate chemical reactivity was assumed. Similarly,  $N_m$  exhibited peak values under several circumstances. The magnitudes of these peaks were affected by ventilation rate but were not particularly sensitive to age.

Although the physicochemical parameters used in the analysis were appropriate for  $\text{O}_3$ , other gases would behave in a qualitatively similar way. The diffusion coefficient for  $\text{O}_3$  is, in

**TABLE 7**  
 Estimated Overall Mass Transfer Coefficient  $K_g$  (cm/s) and Percent of the Overall Diffusion Resistance  
 Due to the Mucous Layer (%) for the Conditions of Figure 20

Gen	Age (yr)											
	0		4		8		12		16		18	
	$K_g$	%	$K_g$	%	$K_g$	%	$K_g$	%	$K_g$	%	$K_g$	%
0	0.217	92	0.201	85	0.197	83	0.193	82	0.192	81	0.191	81
1	0.220	93	0.209	88	0.206	87	0.204	86	0.203	86	0.203	86
2	0.225	95	0.218	92	0.216	91	0.215	91	0.214	91	0.214	91
3	0.226	96	0.221	93	0.219	93	0.218	92	0.217	92	0.217	92
4	0.227	96	0.224	95	0.224	95	0.223	94	0.223	94	0.223	94
5	0.229	97	0.225	95	0.224	95	0.224	95	0.224	95	0.224	95
6	0.230	97	0.226	96	0.225	95	0.225	95	0.224	95	0.224	95
7	0.236	97	0.229	96	0.228	96	0.228	96	0.227	96	0.227	96
8	0.248	97	0.235	97	0.232	96	0.231	97	0.231	97	0.231	97
9	0.260	97	0.242	97	0.238	96	0.236	96	0.234	96	0.233	96
10	0.278	97	0.259	97	0.252	97	0.248	97	0.245	97	0.244	97
11	0.307	97	0.290	97	0.284	97	0.279	97	0.275	97	0.273	97
12	0.326	97	0.308	97	0.301	97	0.295	97	0.291	97	0.290	97
13	0.357	97	0.341	97	0.335	97	0.329	97	0.324	97	0.323	97
14	0.393	97	0.379	97	0.374	97	0.369	97	0.364	97	0.363	97
15	0.439	97	0.430	97	0.426	97	0.423	97	0.420	97	0.419	97

*Note.*  $K_g$  computed from individual gas and liquid phase coefficients as suggested by Treybal (1980). Gen=airway generation of a symmetrically bifurcating tracheobronchial tree. Gen 0 is the trachea and Gen 15 corresponds to the terminal bronchioles.

fact, similar to other reactive gases of environmental concern.  $O_3$  is sparingly soluble, however, and highly soluble/reactive gases such as chlorine and formaldehyde would undoubtedly be absorbed more in the URT and more proximally in the lungs.

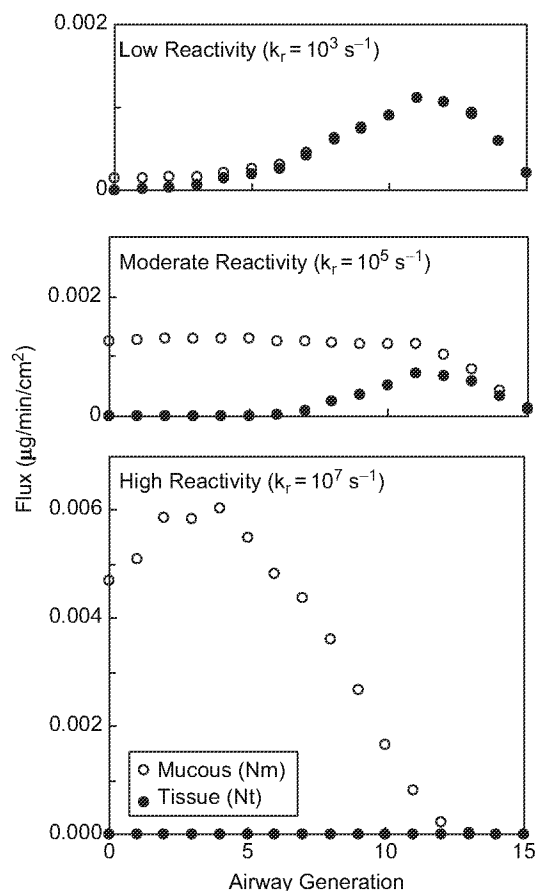
It is important to point out the limitations associated with the current work. First, although this analysis focused on the tracheobronchial tree, the upper airways remove a substantial amount of inhaled reactive gas before it reaches the lower airways. Second, the airway lumen model only incorporates uptake during steady state inspiratory flow. Thus, it does not consider uptake during expiration and can not account for the effects of tidal volume or breathing frequency. Third, because the reaction rate of the inhaled gas is assumed to be first order, the mucus layer model can not accommodate situations where the availability of substrate becomes a limiting factor in the diffusion-reaction process. Fourth, the use of mass transfer correlations for straight tube flow only provides a rough approximation of the convective-diffusion occurring in the lumen of bifurcating airway branches. Fifth, the estimates of mucus thickness as a function of age were rudimentary. It was assumed that mucus thickness scales with airway diameter when it may really depend on other factors more closely associated with the dynamics of the mucociliary system. Finally, our simulations on a specific airway model were completed based on a limited number of airway casts (Phalen, et al., 1985).

In spite of these limitations, the analysis revealed some important trends in the uptake distribution of a reactive gas. Under the current assumptions of anatomy and air flow, children do not appear to receive markedly different mucus or tissue flux than adults. Critical gaps remain to be filled, such as our knowledge of chemical reaction rates and mucus thicknesses in the developing lung.

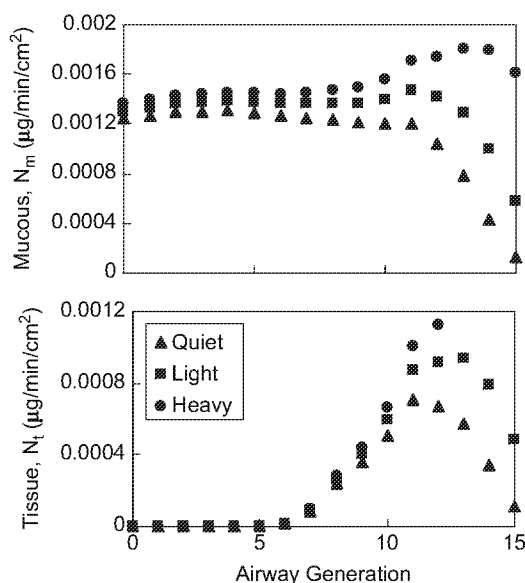
#### NASAL IMAGING AND COMPUTATIONAL FLUID DYNAMICS-BASED DOSIMETRY MODELS TO REFINE RISK ASSESSMENTS FOR CHILDREN

Refining children's inhalation dosimetry estimates will be aided by models that simulate air flow based upon detailed three dimensional (3D) anatomical descriptions of the airways. Computational fluid dynamic (CFD) models were developed as a tool to explore respiratory tract deposition of particles and aerosols in lab animals and adult humans, but efforts to adapt this methodology to children is just beginning. This section describes some of the current applications of CFD modeling and its potential to inform risk assessment for children.

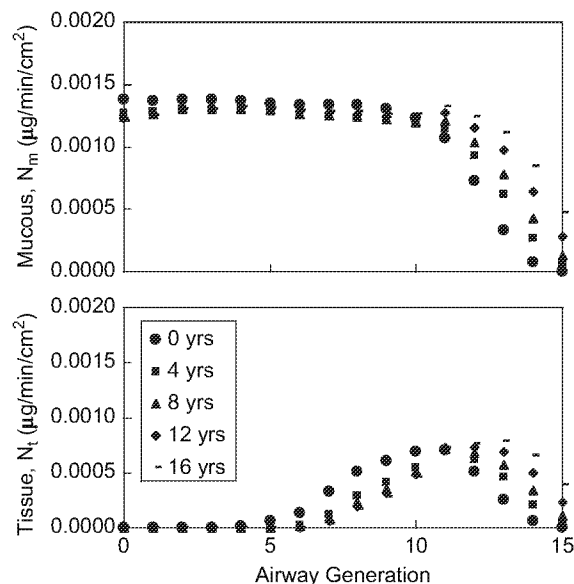
CFD models are composed of an anatomically-accurate grid or mesh of the airways that is used to solve the equations of inhaled air and material transport as shown for the URT in Figure 22. These solutions allow prediction of localized dose



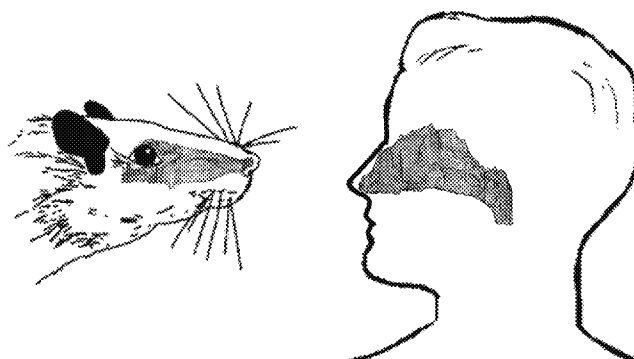
**FIG. 19.** Prediction of flux to the lower respiratory tract for an 8-yr-old inhaling 0.1 ppm of a gas of varying reactivities under quiet breathing conditions.



**FIG. 20.** Estimates of ozone flux to the lower respiratory tract for an 8-yr-old inhaling 0.1 ppm  $O_3$  at various activity levels (Table 5) assuming a moderate reactivity with mucus ( $k_r=10^5 \text{ s}^{-1}$ ).



**FIG. 21.** Flux of ozone to mucus (top panel) and tissue (bottom panel) predicted for children of different ages inhaling 0.1 ppm  $O_3$  during quiet breathing. Assumption of moderate reactivity with mucus ( $k_r=10^5 \text{ s}^{-1}$ ).



**FIG. 22.** Lateral views of the three-dimensional meshes from computational fluid dynamics (CFD) models of the nasal passages of an adult male rat (left) and human (right). (Head outlines provided for reference.)

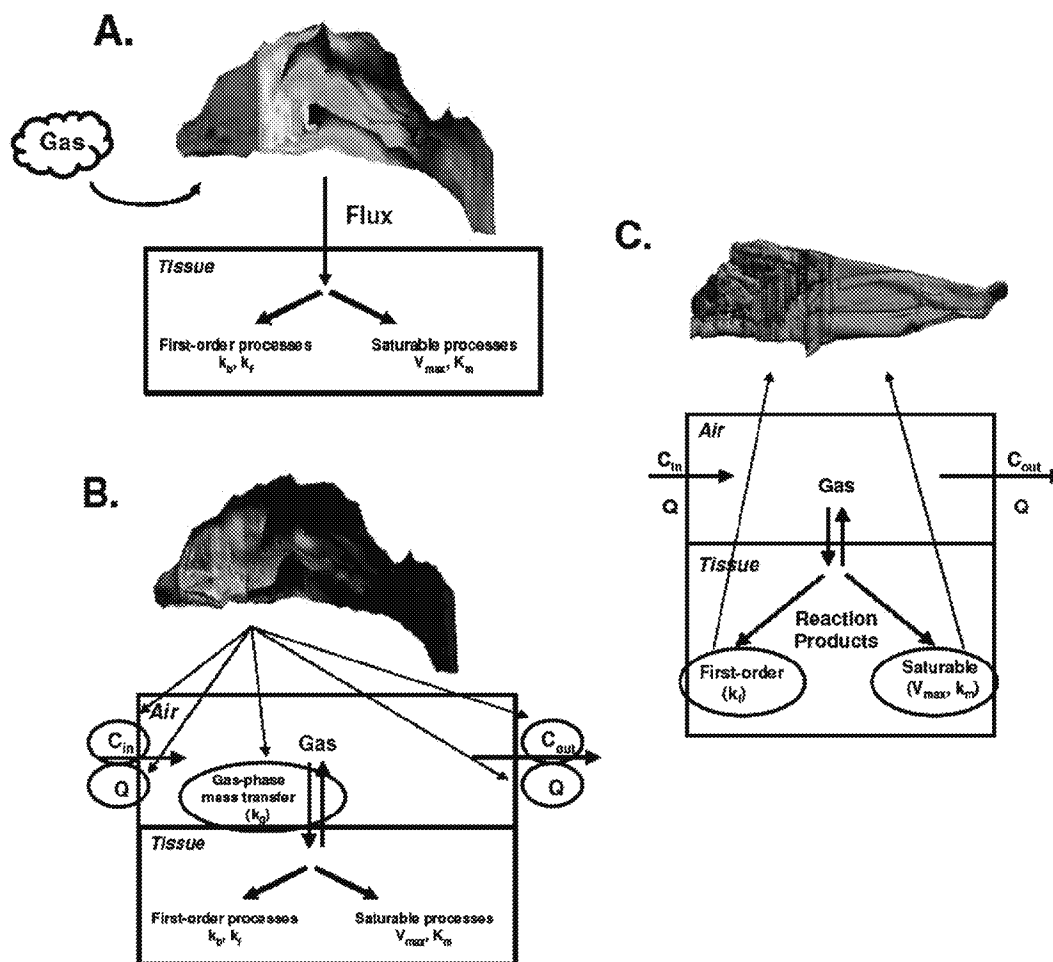
of inhaled material to airway walls (Kimbell et al., 2001a; Moulin, et al., 2002). CFD airway geometry is usually derived from computer-aided tomography (CT) scans, magnetic resonance imaging (MRI) scans, or digital photographs of microscope slides or sectioned specimens. The description of the walls of the CFD model includes spatial information on the distribution of epithelial types and metabolic activity. This information influences the predicted uptake and distribution of inhaled material and aids in interpreting modeling results. The CFD model may also be linked to a physiologically-based pharmacokinetic (PBPK) model of tissue disposition if the

mode of action suggests that including this detail is necessary for accurate prediction of the dose associated with important toxicity. PBPK models typically represent regions of the body as well-mixed compartments based on regional air and tissue volumes and may also encode information on biochemistry, metabolism, and the flows of air, blood and other body fluids to predict dose within compartments.

CFD models of the nasal passages were linked to PBPK or other dosimetry models in three ways. First, a CFD model for air-phase transport provides input to a PBPK model for tissue transport (Figure 23A). An example of this type of linkage is the estimation of formaldehyde-induced DNA-protein cross links (DPX) in the rat and human nasal passages. Here CFD models were used to predict regional wall mass fluxes of formaldehyde. These predicted fluxes were used as inputs to PBPK models that in turn predicted DPX formation (Cohen Hubal et al., 1997; Conolly et al., 2000). Nasal CFD uptake predic-

tions were also used to calibrate the estimated flux from the nasal compartment to the lower regions of single-path mass transfer models of the entire respiratory tract at different flow rates. The calibrated respiratory tract model was used to predict formaldehyde uptake in the human lungs for various activity patterns (Overton et al., 2001).

Second, CFD models were used to provide some of the parameter values of a PBPK model (Figure 23B). Examples of such CFD-informed PBPK models include the transport of acidic vapors (Frederick et al., 1998) and methyl methacrylate (Andersen et al., 1999). In these models, CFD-derived air-phase mass transfer coefficients that estimate the amount of resistance encountered by a gas as it passes from the bulk airstream to the airway walls were incorporated into the transport process from air to tissue compartments. Third, PBPK models are used to optimize parameters that are then used in the airway wall boundary conditions of CFD models



**FIG. 23.** Linkages between CFD and PBPK models. (A) CFD model predicts flux of inhaled material from the air-phase directly into a PBPK model for tissue transport. (B) CFD model is used to calculate parameter values that are then used in a PBPK model. (C) PBPK model of a rat is fitted to nasal extraction measurements to optimize the values of parameters that are then used in the airway wall boundary conditions of a rat nasal CFD simulation.

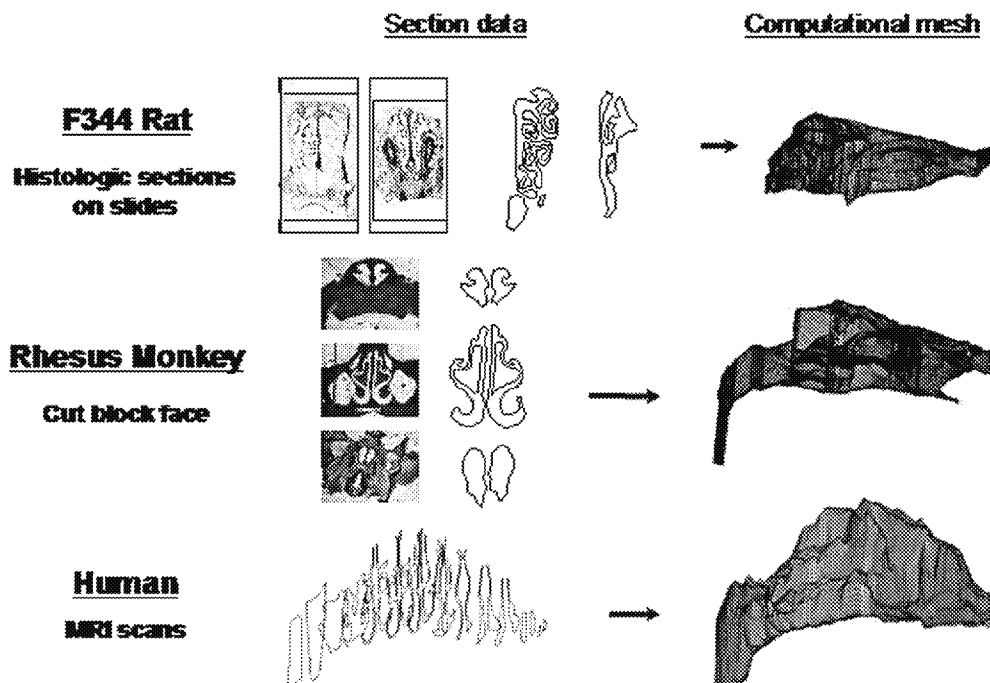
(Figure 6–2C). The model for hydrogen sulfide transport developed by Schroeter and colleagues (2006a;2006b) is an example of a PBPK-informed CFD model. First-order and saturable metabolic parameters were obtained by fitting a PBPK model for nasal extraction of hydrogen sulfide to data measured in rats. The fitted parameters were then incorporated into the airway wall boundary conditions of a CFD model for hydrogen sulfide transport in the air phase so that localized wall mass flux predictions could be compared with lesion distribution data. Nasal CFD models were created for many species including frogs, rats, rabbits, horses, monkeys, and adult humans.

Figure 24 shows how different types of anatomical information were used as the basis of CFD models for rats, monkeys and humans. The CFD model develops airflow patterns from the anatomical mesh, and when combined with mathematical descriptions of boundary conditions (air/mucus interface) and chemical-specific diffusivity and mass transfer coefficients, leads to estimates of gas uptake and particle deposition (Figure 25). Nasal CFD models are useful in risk assessment for testing hypotheses about dominant mechanisms of toxicity (Hotchkiss et al., 1994; Cohen Hubal et al., 1996; Kimbell et al., 1997; Moulin et al., 2002; Schroeter et al., 2006a), for extrapolating tissue responses in lab animals to individuals on the basis of tissue dose (Conolly et al., 2000;2002;2004;

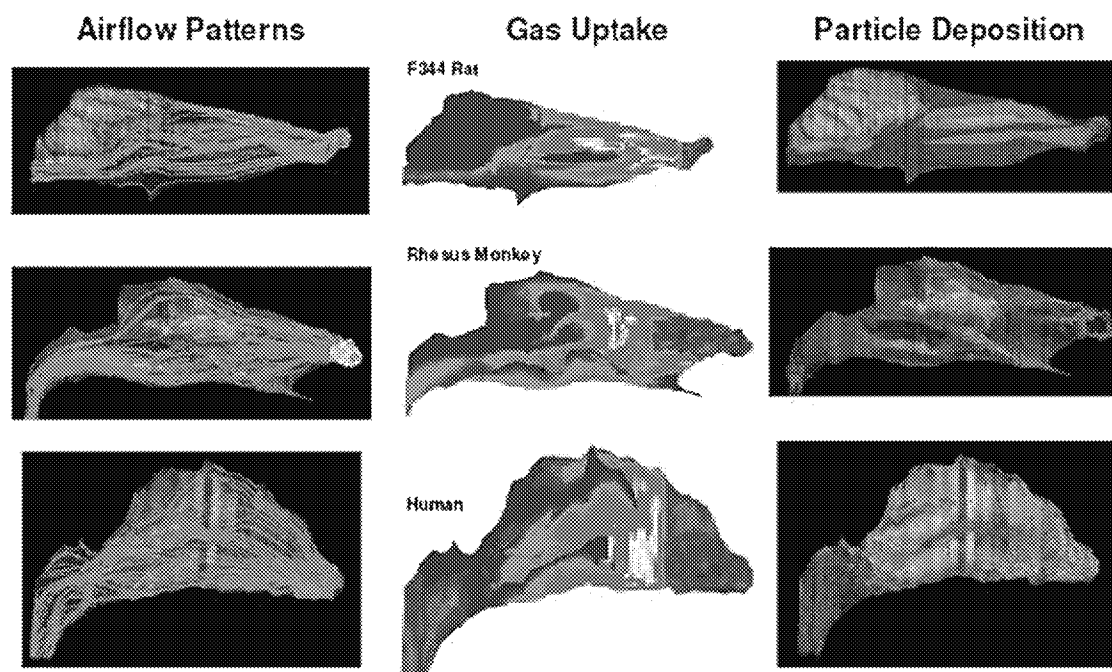
Kimbell et al., 2001b; Schroeter et al., 2006b), and for exploring effects of interindividual variability in nasal geometry on dose among adult humans (Segal et al., 2004; Kimbell et al., 2005a). The need to include children in the study of inter-human dose variability has motivated the extension of nasal imaging-based modeling to early life stages.

A first attempt to incorporate age-specific anatomical and breathing parameters into 3D nasal dosimetry was made by scaling an adult nasal CFD model by nasal volume to represent other age categories (Kimbell et al., 2005b). In this study, age-specific ventilation rates for various activity states and hr/day in the activity (Table 8) were used to predict localized nasal uptake of inhaled formaldehyde for 5 age groups: 3 months, 1 year, 5 years, 10 years, and 15 years old. Together with formaldehyde uptake predictions for age-specific lung generations and activity pattern information, these results will be used in a clonal growth model for formaldehyde carcinogenesis to estimate lifetime cancer risks and compared with lifetime risk estimates that were based on adult dosimetry predictions for all life stages.

Improvements in CT and MRI scanning technologies made it possible to make high-resolution images of the nasal passages of children on which CFD models for children's nasal dosimetry models may be based (Figure 26). However, scans are relatively rare in children, especially infants, and archived scans are often of low resolution and thus of limited utility.



**FIG. 24.** Nasal CFD model construction. Examples of anatomical data upon which nasal CFD models have been based. For the rat and monkey nasal models shown here, airway outlines on slides of tissue sections (rats) or tissue block faces (monkey) were photographed and processed by hand-tracing (rat) or image analysis (monkey). For the human model shown here, MRI or CT images were used. New imaging technologies are making MRI and CT images possible even for the small, intricate nasal passages of mice and rats. Modified from Kimbell (2006).



**FIG. 25.** CFD dosimetry computation. Examples of CFD modeling results. Predicted patterns of inspiratory airflow are illustrated by streamlines (left panels). Regional predictions of inhaled formaldehyde uptake (Kimbell et al., 2001a) show highly nonuniform patterns (center panels; red indicates high uptake rates; blue indicates low uptake rates; reprinted from Kimbell et al. (2001a) with permission from Oxford University Press. Estimates of localized nasal particle deposition can aid understanding of interspecies differences in responses to particle exposure (right panels; modified from Kimbell (2006).

**TABLE 8**  
Age-Specific Activity Patterns Used in CFD Modeling of Formaldehyde Deposition

Age	Sleeping		Sitting		Light exercise		Heavy exercise	
	$V_E$ (L/min)	h/d	$V_E$ (L/min)	h/d	$V_E$ (L/min)	h/d	$V_E$ (L/min)	h/d
3 mo	1.5	17			3.2	7	NA	NA
1 yr	2.5	14	3.7	5	5.8	5	NA	NA
5 yr	4.0	12	5.3	3	9.5	6	37.0	3
10 yr	7.0	10	8.0	3	23.0	8	48.7	3
15 yr	7.5	10	9.0	4	25.0	7	50.0	3
Adult <sup>b</sup> (not at work)	7.5	8 <sup>a</sup>	9.0	8 <sup>a</sup>	25.0	8 <sup>a</sup>	NA	NA
Adult(light work)	7.5	8 <sup>a</sup>	9.0	6 <sup>a</sup>	25.0	9 <sup>a</sup>	50.0	1 <sup>a</sup>
Adult(heavy work)	7.5	8 <sup>a</sup>	9.0	4 <sup>a</sup>	25.0	10 <sup>a</sup>	50.0	2 <sup>a</sup>

Note. Source: ICRP (1994). = minute ventilation (cyclic breathing). NA=not applicable.

<sup>a</sup>Adult is defined as male at 21 yr.

<sup>b</sup>Conolly et al. (2004), based on ICRP (1994).

Improved imaging data for children's nasal airways is critical for developing a reliable CFD approach for predicting toxicant uptake evaluating inter-individual variability.

Advances in imaging technology and CFD model construction are leading the way toward more complete anatomical

descriptions and models of the entire respiratory tract (Corley et al., 2006). Ongoing work in 3 and 6-month old non-human primates involves the development of 3D reconstructions of lung and nasal airways to enable localized analysis of O<sub>3</sub> dose (Carey et al., 2007). The deposition patterns obtained from this



**FIG. 26.** A three-dimensional reconstruction from CT scans of the outer head and nasal passages of a 14-yr-old human female.

modeling will be used to predict the main loci of ozone injury and help understand the effects of air pollution on the developing respiratory tract. Scanning anatomical data in humans of different ages would enable model extrapolation of dose response seen in animals to humans.

The development of children's CFD models will allow us to (1) make localized predictions of dosimeters, (2) study the effects of interindividual variation in anatomy and breathing parameters, and (3) make direct comparisons of dose among species and life stages. The combined CFD/PBPK modeling approach that has been successful for prediction of adult inhalation dosimetry will have similar advantages in children: multiscale levels of dose resolution including cellular, organ and system levels, and the ability to base risk estimates on relevant, species-specific dose predictions for dose metrics motivated by the mode of action. Combined with increased information on anatomical and metabolic data for children's respiratory tracts such as mucus and tissue thicknesses, cell type and enzyme activity distributions, and localized blood flow and biochemical reaction rates, image-based CFD/PBPK modeling will significantly improve the scientific basis for accurate inhalation risk estimation in children.

## DECISION ANALYSIS FRAMEWORK FOR COMPARING DOSIMETRY MODELS

Both the fact that this workshop was convened, and a review of the topics covered, demonstrate that advances in biotechnology drive regulatory authorities such as the U.S. EPA to keep risk assessment approaches contemporary with the state of the science. To do so, the goal of regulatory risk assessment is to integrate diverse types of data now becoming available (e.g., functional genomics) with established outcome measures of adverse health effect, typically endpoints observed at the population (e.g., mortality and morbidity), target tissue (e.g., organ histopathology), or subcellular levels (e.g., clinical chemistry). The challenge to integration will be to rectify these

observations at the more microscopic level of organization with traditional default notions about the shape of the dose-response relationship and to appropriately modify approaches for dosimetry descriptions and associated interspecies and intrahuman variability. For example, the U.S. EPA has revised its cancer risk assessment guidelines to emphasize the use of as much mechanistic data as possible to identify the mode of action (MOA), defined as the influence of a chemical on molecular, cellular and physiological functions, in producing toxicity (U.S. EPA, 2005).

However, the characterization of the MOA and estimation of risks to children from air pollutants is complicated by the lack of reliable age-specific epidemiological data. Extrapolations from adults or lab animals present significant challenges: (1) differences in the MOA due to pharmacokinetic (PK) or pharmacodynamic (PD) differences; (2) integration of data ranging from *in vitro* biochemical to population studies; and (3) analysis of the quality and reliability of predictions. As discussed in the introduction, risk assessment approaches address these issues in a somewhat piecemeal fashion, ranging from default UF for interspecies and intrahuman variability to the use of mechanistic models. Pharmacokinetic (PK) and PD data are being developed for different life stages in lab animals and biomonitoring advances may provide measurements directly in humans (both adults and children). While these new technologies bring MOA data to bear on extrapolations, the problem of comparing disparate models that may use the same or different aspects of the information remains. For example, one dosimetry model may have a different range than another (e.g., see Section 3 for particle dosimetry models). As another example, one dosimetry model may empirically estimate age-specific doses at the population level while another predicts doses at the tissue level using mechanistic descriptions of processes believed to be involved. This latter case is essentially the difference illustrated for modeling reactive gas uptake between the default "rudimentary" predictions of parent uptake described in Section 3 versus calculations of tissue dose metrics described in Sections 4 and 5. Often when evaluations of models are made it is not recognized that different dose metrics are being compared.

Ultimately a synthesis of diverse data is needed to arrive at decisions regarding (in the case of our specific application here) the utility of a dosimetry model structure to provide a dose estimate for use in dose-response analysis. This has placed emphasis on arriving at decisions in a rational and reliable manner (National Research Council, 1994). Decision analysis tools are proving useful to articulate principles underlying MOA in order to formalize an approach that may be used to judge the rationality and reliability of different model structures. This judgment is within the broad arena of how the resultant risk estimates from a model are to be used or applied under the different conditions required by regulatory risk assessment (Jarabek and Crawford-Brown, personal communication). Considering the target context of a specific model

(e.g., gas uptake in the URT of a 10-year old child) is a critical part of ascertaining whether various premises and parameters used in a model represent an appropriate means to reach the end application. That is, systematic comparison of competing models, their resultant estimates, and their epistemic status (i.e., degree of evidential support) must be done relative the decisions being made based on their application (e.g., screening versus health standard promulgation). A key criterion for constructing this target context is to also acknowledge that it must be an iterative process to allow advances in interpretation and application to evolve with the state of the science, indeed the very motivation for using decision analysis.

The decision analytic framework is proposed to (1) systematically analyze and compare model structures; (2) evaluate data interpretation, integration, and reliability; (3) assess valuation of parameters; and (4) rationally assess the quality of resulting model predictions (Jarabek and Crawford-Brown, personal communication). The proposed approach first develops a conceptual model to identify both the process of interest and the target context (e.g., reactive gas uptake in nasal tissue of 10-year old children). The next step is to identify key parameters involved in calculations that take a given exposure to a dose metric deemed relevant to the process (e.g., gas concentration, ventilation rate, mass transfer, and flux to specific airway epithelium). After identification, the next step is to determine the quality and relevance of the data, parameter values, and extrapolation premises used to support a given model structure and predictions. Issues of data reliability (the extent to which the evidence can be used to form an inference) and relevance (the extent to which the evidence has the tendency to make a fact probable within the context of a specific judgment being formed) represent theoretical concepts relating to the degree of evidentiary support or the level of proof. An important aspect in the approach is that this level of proof may be different as one extends the dose description to different levels of organization.

Criteria to assess these judgments, premises, and parameters are different for different categories of evidence (e.g., direct empirical or theory-based inference) and are listed in Table 9 (Bunge, 1987). These 7 principles are then combined into an application for rational risk analysis (Crawford-Brown, 2005). The framework then assesses the rationality of predictions of the process in a target context (human children), including evaluation of the strengths and weaknesses of alternative parameters and model forms, the reliability of resulting risk estimates, and key sources of residual uncertainty. Such an analysis would be transparent to external scrutiny and show how conclusions follow deductively from premises. The premises are examined for uncertainty and how they propagate through to uncertainty in resultant conclusions. Rational strategies are formalized most clearly by logic trees or inference frameworks, and the steps in the framework are shown schematically in Figure 27 (Jarabek

**TABLE 9**  
Principles of Rationality

- 
- Conceptual clarity: Terms are rigorously defined and agreed upon by the relevant community.
  - Logical consistency: Predictions or estimates follow deductively from assumptions made and the data used.
  - Ontological soundness: Terms appearing in an analysis conform to scientific understanding of the phenomenon (e.g., mass transfer) in question.
  - Epistemological reflection: Assumptions used are subject to scrutiny to determine degree of evidential support.
  - Methodological rigor: Use of clearly defined methods that have proven reliable in past applications.
  - Practicality: Methods can be completed in a reasonable length of time and with reasonable resources.
  - Valuational selection: Attention is focused on values deemed most important.
- 

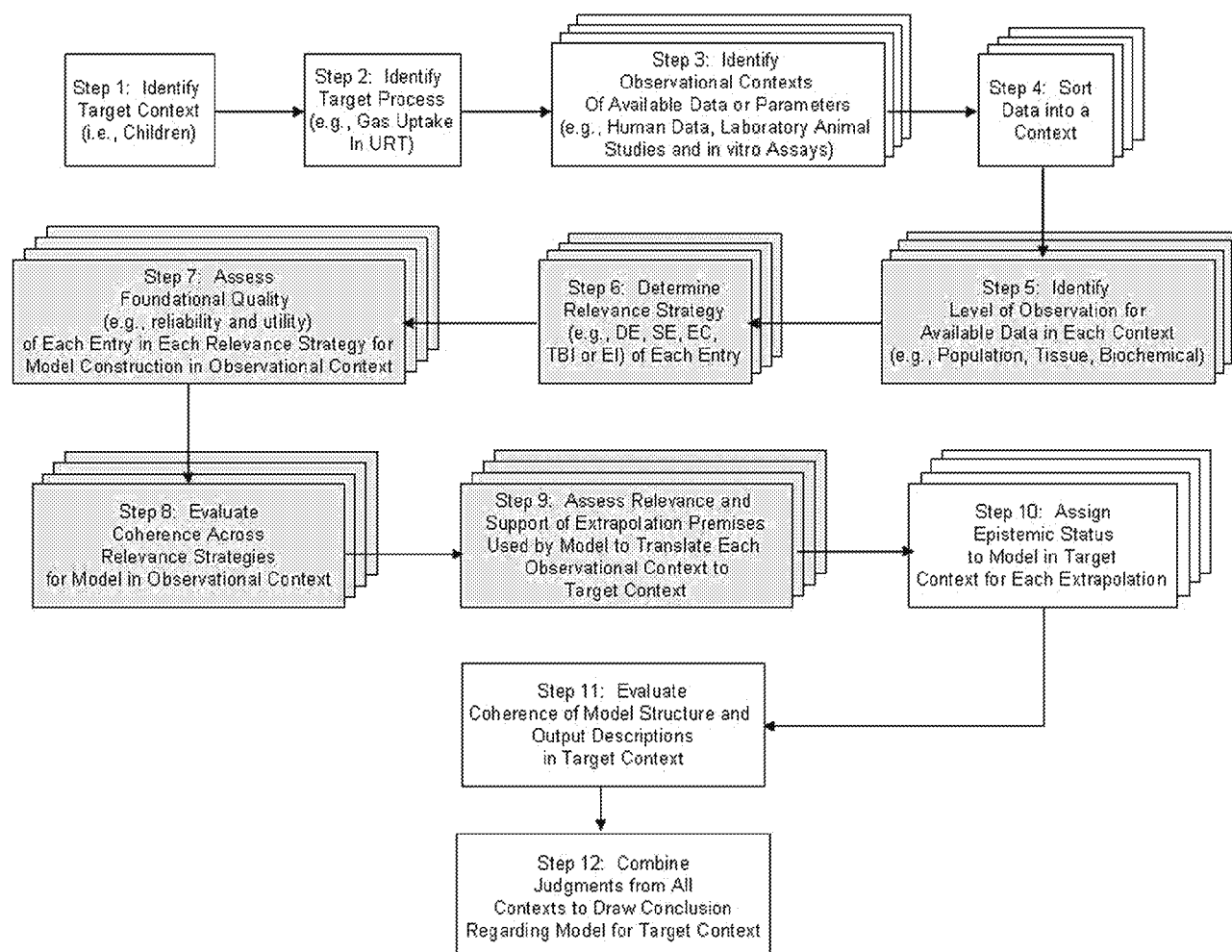
*Note.* Source: Bunge (1987).

and Crawford-Brown, personal communication). If needed for the application, a formal uncertainty analysis is facilitated by this analysis tree.

Using the decision approach to compare model structures developed to address children's risk will aid their systematic evaluation. Because the process diagrams show the flow of information to arrive at a decision, issues of reliability regarding premises are readily revealed. Critical parameter values that may need more data are also easily pointed out.

#### **SESSION SUMMARY: APPLYING CHILDREN'S INHALATION DOSIMETRY MODELING IN HUMAN HEALTH RISK ASSESSMENT**

The model structures and simulations presented in this session of the workshop covered a wide range of inhaled materials (ultra-fine, fine, and coarse particles; reactive to non-reactive gases), age groups (3 months to adult), and modeling types (default to data-intensive CFD models). Despite limitations in available age-specific data, the overall conclusion is that there are important anatomical and physiological differences between young children and adults that may lead to important differences in internal dose of these inhaled materials. Differences in delivered dose may account for observed differences in susceptibility of children versus adults (Bobak and Leon, 1999; Gent, et al. 2003). This may be especially true for particle deposition in the lower respiratory tract where an increased dose relative to adults may lead to enhanced adverse pulmonary effects and mortality in children (Bobak and Leon, 1999). Thus, factors that determine inhalation dosimetry need to be



**FIG. 27.** Schematic of general steps involved in a rational decision analysis for comparison of different dosimetry model structures. The stacked boxes indicate different observation contexts (e.g., different laboratory or species). The shaded boxes denote where data may need to be considered at various levels of observation (e.g., target tissue, cellular, and biochemical) and different dose descriptions may occur at each level. Relevance strategies (evidence categories) listed in step 4 are depicted as DE=direct empirical, SE=semiempirical, EC=empiric correlation, TBI=theory-based inference, and EI=existential insight. Adapted from Crawford-Brown (1999) and Jarabek and Crawford-Brown (personal communication).

considered in addition to behavioral factors (time spent in physical exertion/play activities, location of these activities) in order to refine estimates for human risk assessments of inhaled materials.

However, children's inhalation dosimetry is not typically estimated or taken into account in risk assessment of inhaled agents. As described in the summary for Session 1 of this same workshop (Foos et al., 2007), data available in some risk assessment processes, such as promulgation of the National Ambient Air Quality Standards (NAAQS), may allow evaluation of children's risk directly. However, in most cases, lab animal data are extrapolated to estimate human health risk. Some approaches that rely on lab animal data do not provide for inhalation dosimetry adjustment to arrive at human estimates, while approaches such as the RfC methods which do

apply dosimetry models, do not explicitly address how to adjust dosimetry to account for physiological and anatomical differences of young children. Instead the potential variability is believed to be addressed by UF applied for both database completeness and intrahuman variability. Thus, potential differences in delivered dose for children predicted by simulation studies such as those discussed in this session need to be evaluated in the context of determining the adequacy of these types of UF applied in most operational derivations to arrive at human health risk estimates.

Although limited in scope, the modeling approaches described in this paper may help inform considerations for adjusting existing RfC or deriving a life-stage specific RfC. Exactly how early life dosimetry differences that exist for one or several years are averaged over the longer lifetime to

which the RfC estimates apply may require a case-specific determination based on the critical effect. For example, as indicated by some of the analyses, there may be larger differences between deposition for some ages of children (e.g., infant to a 6-year old) than between other ages of children and adults (e.g., 12-year old and 21-year old). If unique windows of susceptibility for a specific disease coincide with those ages that also may receive higher doses, then a life stage specific RfC may be needed. The magnitude of the difference in dosimetry needs to be evaluated in context with that of the intrahuman UF and other UF that apply (e.g., database deficiencies). In contrast, if the relevant period of inhaled toxicant exposure is many years, then any higher dosimetry at an early life stage may need to be time-averaged with other age groups to adjust the RfC. In either case, it is important to document to what extent children's inhalation exposures have been considered when evaluating community exposures to inhaled toxicants.

The hierarchical model framework provided in the 1994 RfC methods (See Section 1 above) may be used to motivate new refinements that address age-specific considerations discussed in this manuscript. To implement such hierarchical considerations, suites of models were proposed to provide the flexibility to describe dose estimates with different levels of detail (Jarabek, 2000). The dose metric needs to be described at a level of detail that is commensurate with both the level of detail available regarding the toxic response and the intended duration of exposure for which the dose-response relationship is derived (U.S. EPA, 1994; Jarabek, 1995b). Thus, just as the preferred models for adults include species-specific and mechanistic determinants of chemical disposition and effect (Jarabek, 1995b; Bogdanffy and Jarabek, 1995), age-specific parameters and models such as those described in this session should be used. For example, in a given risk assessment the preferred model may take the form of a single-path mass transfer model to describe regional gas uptake (see Section 3 above), involve more refined multi-pathway deposition models (see Section 4 above), or be enhanced by chemical-specific information on reactions in the mucus layer (Section 5 above), metabolism in airway epithelium, and interaction with critical target molecules (e.g., the formaldehyde example in Section 6 above). All of these options should incorporate children's parameters to the extent possible.

## DATA NEEDS

While techniques exist to develop a suite of modeling options, the limiting factor is having data of sufficient quality and reliability to support the required key parameters. The lack of age-specific anatomical and ventilation rate data is considered the key information gap. Additionally, the following are identified as data needs for children's inhalation dosimetry:

- A database able to support such a suite of models with sufficient sophistication and flexibility will require

the compilation of an anatomical database for respiratory tract regions across a range of age groups (informed by imaging techniques, airway cast measurements, digital photographs of microscope slides or sectioned specimens, and scaling techniques).

- Age-specific clearance rates from respiratory regions.
- Breathing mode (normal augmentation versus mouth breathing), and the influence of ventilatory activity patterns on the relative contribution of nasal versus oral breathing, were shown to be key determinants of initial particle deposition and gas uptake (Snipes et al., 1997; Conolly et al., 2002).
- Determination of the switching point from nasal only to oral augmentation is likely to be different in children as discussed by Bennett et al. in a companion paper (Foos, et al., 2007); this will be an important consideration when using children's ventilation rate data.
- Physical attributes (e.g., particle size and distribution) and other physicochemical properties (e.g., composition and hygroscopicity) are also important and warrant characterization to refine models in both adults and children. For example, particle hygroscopicity influences inhaled deposition (ICRP, 1994; Schroeter et al., 2001).

Addressing these physicochemical, anatomical, and physiological data gaps will be key steps in building a suite of comprehensive dosimetry models for inhaled materials in both adults and children. Further, development of a comprehensive modeling capability across age groups may also decrease the reliance on default UF currently applied to account for intrahuman and database deficiencies.

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# Quantitative Approach for Incorporating Methylmercury Risks and Omega-3 Fatty Acid Benefits in Developing Species-Specific Fish Consumption Advice

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**BACKGROUND:** Despite general agreement about the toxicity of methylmercury (MeHg), fish consumption advice remains controversial. Concerns have been raised that negative messages will steer people away from fish and omega-3 fatty acid (FA) benefits. One approach is to provide advice for individual species that highlights beneficial fish while cautioning against riskier fish.

**OBJECTIVES:** Our goal in this study was to develop a method to quantitatively analyze the net risk/benefit of individual fish species based on their MeHg and omega-3 FA content.

**METHODS:** We identified dose-response relationships for MeHg and omega-3 FA effects on coronary heart disease (CHD) and neurodevelopment. We used the MeHg and omega-3 FA content of 16 commonly consumed species to calculate the net risk/benefit for each species.

**RESULTS:** Estimated omega-3 FA benefits outweigh MeHg risks for some species (e.g., farmed salmon, herring, trout); however, the opposite was true for others (swordfish, shark). Other species were associated with a small net benefit (e.g., flounder, canned light tuna) or a small net risk (e.g., canned white tuna, halibut). These results were used to place fish into one of four meal frequency categories, with the advice tentative because of limitations in the underlying dose-response information. Separate advice appears warranted for the neurodevelopmental risk group versus the cardiovascular risk group because we found a greater net benefit from fish consumption for the cardiovascular risk group.

**CONCLUSIONS:** This research illustrates a framework for risk/benefit analysis that can be used to develop categories of consumption advice ranging from "do not eat" to "unlimited," with the caveat that unlimited may need to be tempered for certain fish (e.g., farm-raised salmon) because of other contaminants and end points (e.g., cancer risk). Uncertainties exist in the underlying dose-response relationships, pointing in particular to the need for more research on the adverse effects of MeHg on cardiovascular end points.

**KEY WORDS:** cardiovascular risk, fish advisory, methylmercury, neurodevelopment, omega-3 fatty acids, risk/benefit. *Environ Health Perspect* 117:267–275 (2009). doi:10.1289/ehp.11368 available via <http://dx.doi.org/> [Online 3 September 2008]

A decade ago, the landmark studies from the Seychelles (Davidson et al. 1998) and Faroe islands (Grandjean et al. 1997) were unfolding and a debate was raging over how much risk is associated with methylmercury (MeHg) in fish. Both the Seychelles and Faroe studies involved populations that have a high per capita consumption of fish and MeHg body burdens generally higher than in the United States (Davidson et al. 1998; Grandjean et al. 1997). The Seychelles study showed no evidence of harm, whereas the Faroe study, at similar MeHg exposure levels, showed significant neurodevelopmental deficits at birth and into the early school years (Axelrad et al. 2007). Interpretation of these studies by the U.S. Environmental Protection Agency (EPA) and Agency for Toxic Substances and Disease Registry (ATSDR) differed, creating confusion in federal and state government over how to set fish consumption advice (ATSDR 1999; U.S. EPA 2001). A National Academy of Sciences report [National Research Council (NRC) 2000] helped resolve the debate by concluding that MeHg in fish is an important public health risk and developed a dose-response analysis for neurodevelopmental effects that was subsequently used by the U.S. EPA to derive the reference dose (RfD) (U.S. EPA 2001). The Seychelles study, although

still overall a negative (without effects) study, recently found some evidence suggestive of a latent MeHg effect (Davidson et al. 2006). An ongoing study of a birth cohort in Massachusetts shows an association of MeHg exposure with neurodevelopmental effects at lower levels of exposure than in prior studies (Oken et al. 2005, 2008).

One might assume that the controversy is over. The issue has been through the National Academy of Sciences, and public health officials now have an RfD on the U.S. EPA's Integrated Risk Information System (IRIS) database (U.S. EPA 2001) that can be used to set fish consumption limits. Why, then, is the subject of fish consumption still as debatable now as it was a decade ago? The answer is that the nutrients in fish, especially the fish oil omega-3 fatty acids (FAs) eicosapentaenoic acid (EPA; C<sub>20:5</sub> n-3) and docosahexaenoic acid (DHA; C<sub>22:6</sub> n-3) have been increasingly identified as having public health benefits. This leads to the concern that avoiding fish because of contaminants will eliminate the benefits from fish consumption, a concern heightened by the fact that the most abundant natural source of EPA and DHA is fish (Racine and Deckelbaum 2007). Balancing the risks and benefits of fish consumption has become an increasingly important goal of fish

consumption advisories. However, recent messages in the media that emphasize fish benefits have created confusion about the need for caution (Hobson 2006). In one case, an advocacy group recommended that pregnant women exceed federal fish consumption guidelines, but that group has subsequently been found to have dubious funding sources (Couzin 2007). On the other hand, warnings about MeHg levels in fish can provide overly negative messages that cause women to completely avoid fish (Cohen et al. 2005a; Oken et al. 2003).

In this article we quantitatively address key aspects of the fish risk/benefit issue by analyzing the health trade-offs for individual fish species. Although MeHg and omega-3 FA are both present in fish, species can be distinguished based on the relative proportion of these constituents (Mahaffey et al. 2007; Stern 2007). The present analysis provides a quantitative approach for identifying which fish are most beneficial for neurodevelopmental and cardiovascular outcomes. Our focus is on the potential utility of the approach rather than the exact results obtained to date, because uncertainties in the underlying dose response make the conclusions tentative. Although showing possible directions for species-specific advisories, the analysis points to key research areas for improving risk/benefit analysis for fish consumption. The demonstrated approach may serve as a model for analyzing fish species, contaminants, and end points not included in the present analysis.

**Evidence of fish consumption effects on neurologic and cardiovascular outcomes.** The ingestion of fish or fish oils has been associated with an array of health benefits, including improvement of blood lipid profiles, decreased risk of heart disease, and lowered blood pressure [Institute of Medicine (IOM) 2006; Mozaffarian and Rimm 2006], improvement in rheumatoid arthritis (Kremer 2000), enhanced eye and brain development in early life (Fleith and Clandinin 2005), prevention of macular

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degeneration (SanGiovanni et al. 2007), lower risk of colitis (Hudert et al. 2006) and type 2 diabetes (Barre 2007), and improvement in neurologic and psychological disorders such as depression, schizophrenia, and Parkinson disease (Calon and Cole 2007). Diets rich in omega-3 FA increase the ratio of omega-3 to omega-6 (primarily from vegetable sources) in cell membranes. This, as well as a host of related effects on lipid chemistry, leads to a generalized antioxidant, anti-inflammatory effect that has documented benefits in neural tissues, vascular endothelium, and cardiac muscle (antiarrhythmic effect) (Connor 2000; Farooqui et al. 2007; Massaro et al. 2006; Mozaffarian and Rimm 2006; von Schacky 2006).

The purported benefits of fish oil omega-3 FA are perhaps best documented for cardiovascular end points and enhanced brain development. It is noteworthy that MeHg also has toxic effects in these areas. Therefore, the present analysis focuses on fish consumption risks and benefits on these end points. The following sections provide a brief review of pertinent literature in these areas as background for our quantitative species-specific risk/benefit analysis.

**Fish and omega-3 FA effects on cardiovascular end points.** Recent reviews of the cardiovascular benefits from fish and fish oils have focused on mortality from coronary heart disease (CHD; IOM 2006; Mozaffarian and Rimm 2006; von Schacky 2007). Evidence from a combination of 20 different prospective cohort studies and clinical trials has shown a consistent decline in CHD mortality with increasing omega-3 FA intake (EPA + DHA) with an apparent saturation of this benefit at intakes > 250 mg/day (Mozaffarian and Rimm 2006). Below an ingestion rate of 250 mg/day, there was a 14.6% decrease in CHD mortality per 100 mg/day omega-3 FA ingested (95% confidence interval, 8–21% reduction). CHD benefits were strongest for oily fish such as salmon, herring, and sardines relative to leaner fish (cod, catfish, halibut). Although the weight of evidence supports a cardiovascular health benefit from fish oils, not all analyses have found this to be the case (Hooper et al. 2006).

The meaning of the saturation of benefit in the Mozaffarian and Rimm (2006) pooled analysis is unclear because it contains studies in which omega-3 FA ingestion was from fish in some cases and from omega-3 FA supplements in others, with this not clearly segregated in their analysis. Saturation of benefit above 250 mg omega-3 FA intake per day may not be an actual plateau, because as fish ingestion increases, so does the intake of MeHg. The toxicity of MeHg on the same cardiovascular end point may cause a net leveling off of the benefit. Separate evaluation of omega-3 FA supplementation studies is

needed to refine the analysis, but in general, there are fewer of these studies and they were not designed to evaluate dose response (Konig et al. 2005). In one particular case, supplementation of the diet of Japanese adults who have cardiovascular disease with 1.8 g/day EPA yielded a measureable benefit on CHD mortality (von Schacky 2007). Given the high level of fish consumption and therefore the high background of omega-3 FA intake in this population, the added benefit from supplemental fish oil suggests that the benefit does not saturate. If this is true, the apparent saturation reported by Mozaffarian and Rimm (2006) may in fact reflect the counterbalancing effect of MeHg. Additional research in this area is needed.

Fish oil may also have benefits on a variety of other cardiovascular end points, including decreases in nonfatal myocardial infarction (MI), ischemic stroke, atrial fibrillation, atherosclerosis, and congestive heart disease (IOM 2006; Mozaffarian and Rimm 2006; von Schacky 2007). However, the evidence in these cases is limited and not currently suitable for a risk/benefit assessment of fish consumption.

**MeHg effects on cardiovascular end points.** MeHg is a risk factor for cardiovascular disease through a variety of mechanisms potentially involving pro-oxidant effects via the generation of radical species and the inactivation of cellular antioxidant systems such as glutathione peroxidase and catalase (Guallar et al. 2002). There is evidence for lipid peroxidation and elevations of oxidized low-density lipoprotein in association with MeHg exposure (Andersen and Andersen 1993; Salonen et al. 1995). Mechanistic studies indicate that MeHg can exert toxic effects on the vascular endothelium by depletion of sulfhydryls, increased oxidative stress, and activation of phospholipases (Hagele et al. 2007; Mazerik et al. 2007). Oral dosing of rats with MeHg at a daily rate of 0.5 mg/kg for 9 months yielded a persistent pressor effect (Wakita 1987), whereas inorganic mercury has caused a variety of adverse effects on cardiovascular function, including increased blood pressure, altered heart rate, and decreased heart contractility (ATSDR 1999). Given that some of these effects occurred at relatively low doses (< 1 mg/kg/day), this appears to be a sensitive target for MeHg's effects. Human overdose with organic or inorganic mercury has also produced a variety of adverse effects on the heart and blood pressure, and occupational exposure to inorganic mercury has been associated with hypertension and nonischemic heart disease (ATSDR 1999; Boffetta et al. 2001).

Epidemiologic evidence is generally supportive of an association between MeHg body burden in the general public, primarily from fish consumption, and cardiovascular disease

(Stern 2005). This database is not as robust as that supporting the benefits of fish oils on CHD, but nevertheless includes substantive findings that need to be accounted for in a risk/benefit analysis. A prospective study of 1,014 Finnish men found that those in the highest quintile of MeHg exposure (hair mercury > 2.81 ppm) had an accelerated thickening of the carotid artery, an indication of atherosclerosis (Salonen et al. 2000). Several studies provide evidence of increased CHD mortality in men in relation to hair or toenail mercury (Guallar et al. 2002; Rissanen et al. 2000; Salonen et al. 1995; Virtanen et al. 2005). In a case-control study spanning eight European countries and Israel, 684 men with MI were found to have significantly greater toenail mercury than the 724 matched controls (Guallar et al. 2002). This association demonstrated a linear dose response that was strengthened when the positive influence of the omega-3 FA DHA was controlled for in the model. An earlier study of 1,833 Finnish men followed prospectively showed a doubling of risk for MI in the highest tertile of exposure (hair mercury > 2 ppm) (Salonen et al. 1995). A follow-up of this eastern Finland population continued to show a heightened risk of coronary events due to MeHg that was able to offset the positive influence of omega-3 FA (Virtanen et al. 2005).

However, several other studies failed to find a consistent association between mercury body burden and cardiovascular outcomes (Ahlqvist et al. 1999; Hallgren et al. 2001; Yoshizawa et al. 2002). A study of 1,462 Swedish women did not find an association between serum mercury and MI or stroke, but that study focused primarily on mercury exposure via amalgam fillings (Ahlqvist et al. 1999). This appears to have been a significant source based on the strong correlations between serum mercury and number of fillings. There was no assessment of fish ingestion or attempt to factor out the benefit of fish oils on the end points measured. In another Swedish study, involving 78 men and women with MI and 124 controls, red blood cell mercury and plasma EPA + DHA were both found to be negative predictors of MI risk (Hallgren et al. 2001). However, the mercury body burden in this population was much lower than in the Finnish studies, possibly too low to have an adverse effect on its own and thus more likely served as a marker for omega-3 FA ingestion from fish. Interestingly, the subgroup with the highest red blood cell mercury and lowest omega-3 FA levels had an elevated odds ratio, but this was not statistically significant possibly due to the low number (10) in this group. Overall, this study did not have sufficient power to detect an independent effect of MeHg on MI, especially given the low exposures to MeHg in this population. Finally, a large prospective study of U.S. health

professionals collected toenail mercury data from 33,737 men, of whom 470 had an MI during the course of follow-up (Yoshizawa et al. 2002). The overall analysis showed no difference in risk of MI across the quintiles of toenail mercury, but also in contrast to other studies, there was no demonstrable benefit from fish ingestion. Most subjects were dentists, and they were overrepresented in the highest exposure groups (40% in the lowest quintile; 84% in the upper quintile). The authors reported a positive but nonsignificant association of mercury with CHD in a subanalysis that excluded dentists. This may indicate that MeHg from fish ingestion has a greater influence on cardiovascular risk than inorganic mercury from dental amalgams. Although speculative, this would help explain the negative findings in the Swedish women's study described above (Ahlqwist et al. 1999).

Overall, mechanistic evidence and results of animal toxicology, human clinical toxicology, and epidemiology studies support the notion that MeHg can be a risk factor for cardiovascular disease. The strongest epidemiology study in this regard is that of Guallar et al. (2002), which provided separate dose–response functions for MeHg risk and omega-3 FA benefit for the same cardiovascular end point. Therefore, we used this study as one of the core studies for our risk/benefit analysis for cardiovascular end points in men.

**Fish and omega-3 FA effects on neurodevelopment.** Fish oils, and in particular DHA, have been associated with a number of beneficial effects on neurocognitive and ocular function, both in early life and in old age. These associations include increased visual acuity in newborns (Uauy et al. 2003), better scores on neurodevelopmental test batteries (Daniels et al. 2004; Fleith and Clandinin 2005; Oken et al. 2005, 2008), and prevention of a number of neuropsychiatric disorders in adults, including attention deficit disorder, Alzheimer disease, schizophrenia, and depression (Calon and Cole 2007; Young and Conquer 2005). Dietary supplementation with DHA prevented a number of biochemical changes induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in a mouse model of Parkinson disease

(Bousquet et al. 2008). The early-life evidence comes from studies in both preterm and full-term infants, with the benefits more consistently shown in preterm infants. These trials have involved the addition of omega-3 FA to infant formula. Part of the impetus for the early-life studies is the finding that formula-fed babies have less plasma and red cell DHA than do breast-fed babies, leading to the question of whether formula should be supplemented with omega-3 FA (Fleith and Clandinin 2005). The strongest association in the fish oil supplementation studies has been with the development of vision, particularly within the first year of life. In addition, maternal ingestion of fish has been associated with enhanced neurocognitive development in ongoing prospective studies (Daniels et al. 2004; Hibbeln et al. 2007; Oken et al. 2005, 2008). Other nutrients in fish may contribute to the neurodevelopmental benefit. However, the fact that this benefit is demonstrable with omega-3 FA supplementation alone indicates an important role for this nutrient (Cohen et al. 2005b).

The present analysis focuses on the evidence of a neurodevelopmental benefit from maternal fish and omega-3 FA ingestion during pregnancy and, in particular, on one study that adjusted for the developmental deficits induced by the concomitant ingestion of MeHg in the fish (Oken et al. 2005), from which it is possible to develop independent dose–response relationships for omega-3 FA benefit and MeHg risk on the same neurodevelopmental end point. Dose–response relationships for MeHg and omega-3 FA effects on IQ have also been derived from a synthesis of the relevant literature (Cohen et al. 2005a, 2005b, 2005c). These other analyses are consistent with the MeHg/omega-3 FA dose responses obtained from the Oken et al. (2005) study that we used as the basis for the present analysis.

**MeHg effects on neurodevelopment.** As mentioned above, adverse effects of MeHg have been observed in studies of maternal exposure from fish ingestion in relation to postnatal neurodevelopment. Oken et al. (2005) provided a very useful dose response for this effect because they corrected for the

benefit of fish oil ingestion. Several large prospective studies also demonstrate an adverse effect of MeHg, although inconsistencies between them led to considerable debate during the 1990s (Davidson et al. 1998; Grandjean et al. 1997; Kjellstrom et al. 1989). The series of reports from the Faroe Islands are consistent with results from New Zealand in showing an adverse effect of MeHg on neurodevelopment, and this has been judged to outweigh the mostly negative findings from the Seychelles Islands (NRC 2000; U.S. EPA 2001). The epidemiology associations are consistent with an extensive literature in rodents and monkeys demonstrating early-life vulnerability to the neurotoxic effects of MeHg (ATSDR 1999).

## Methods for Integrated Risk/Benefit Analysis

We selected studies from the literature described above to support an integrated risk/benefit analysis for adult cardiovascular and *in utero* neurodevelopmental end points on a species-specific basis. Table 1 summarizes the dose–response relationships found for omega-3 FA and MeHg for common end points: cardiovascular disease in adults (CHD mortality or first MI) and neurodevelopment in 6-month-old infants [visual recognition memory (VRM)]. The adult end points are very similar because both are a measure of coronary artery health; the CHD end point includes fatal MI and sudden death (Mozaffarian and Rimm 2006), whereas the first MI is not necessarily fatal (Guallar et al. 2002). The omega-3 FA benefit on this end point was taken directly from the reported slope for change in relative risk per 100 mg/day intake of EPA + DHA (Mozaffarian and Rimm 2006). This dose response was not adjusted for the countervailing effect of MeHg and so may underestimate the true relationship or suggest a plateau in benefit that is in fact an indication of MeHg toxicity (see above). We estimated the dose response for MeHg effects on MI from Figure 1A of Guallar et al. (2002) based on the relationship between toenail mercury and MI odds ratios. We used the DHA-adjusted slope from Guallar et al. (2002) in the present analysis. Because the odds ratio is often an overestimate

**Table 1.** Dose–response relationships for key MeHg and omega-3 FA end points.

End point	Agent	Dose response	Comments	References
Adult CHD mortality	Omega-3 FA	14.6% decreased relative risk per 100 mg/day	Combined data across 20 studies for EPA + DHA intake versus CHD mortality; possible saturation of benefit > 250 mg/day	Mozaffarian and Rimm 2006
Adult MI risk	MeHg	23% increased relative risk per 1 ppm hair Hg	Slope adjusted for DHA content of lipid as index of fish oil intake; risk not apparent < 0.51 ppm hair Hg; toenail Hg measured but converted to ppm in hair	Toenail to hair Hg conversion, Guallar et al. 2002, Ohno et al. 2007; odds ratio conversion to relative risk, Zhang and Yu 1998
Infant VRM score	Omega-3 FA	2.0-point increase per 100 mg/day	VRM measured at 6 months in 135 mother–infant pairs; fish oil intake estimated from dietary survey	Oken et al. 2005
Infant VRM score	MeHg	7.5-point decrease per 1 ppm hair Hg	VRM measured at 6 months in 135 mother–infant pairs; direct measurement of maternal hair Hg	Oken et al. 2005

of the relative risk and because the omega-3 FA cardiovascular benefit was in terms of improved relative risk (Mozaffarian and Rimm 2006), we converted the Guallar et al. (2002) data to relative risk by the equation provided by Zhang and Yu (1998). This provides a reasonable estimate of relative risk, although a small (15%) relative bias is possible with this method (McNutt et al. 2003).

This dose response for MeHg effects on MI risk has a hair mercury threshold of 0.51 ppm before any adverse effect is evident (Guallar et al. 2002). Although much of the population in that study had mercury levels in this range and below, there was no clear dose-response trend until the body burden rose above this apparent threshold. The appearance of a threshold may be related to measurement error and variability in the baseline population that obscures a mercury effect below that level. If there is a mercury effect on MI at levels < 0.51 ppm in hair, the slope may be different than that seen at higher body burdens. Therefore, our estimate of mercury MI risk includes this threshold but it is a source of uncertainty.

Infant VRM is a common end point for both omega-3 FA and MeHg because these agents had opposite effects in the 135 mother-infant pairs evaluated by Oken et al. (2005). VRM is a test that evaluates an infant's ability to encode a stimulus (photograph) into memory and recognize a new stimulus as novel and preferential to the old stimulus. This test is predictive of IQ at later developmental stages (Rose and Feldman 1995). The slope for the hair mercury effect on VRM score was taken directly from Table 2 of Oken et al. (2005), who adjusted the slope for the amount of fish ingestion. Oken et al. (2005) derived the relationship between

omega-3 FA intake and VRM score from analysis of food survey records and estimation of omega-3 FA content of fish in relation to the VRM score for each individual, with correction for the inverse association with hair mercury (Oken E, personal communication).

We ran the dose-response functions shown in Table 1 in Excel spreadsheets (Microsoft Corporation, Redmond WA) to estimate the effect of one or more fish meals on the outcome measure using the following risk/benefit equations:

$$\begin{aligned} \text{Net risk/benefit for adult CHD} = & \\ & [(\text{omega-3 FA mg/meal}) \\ & \times (\text{no. meals/week}) \times (1 \text{ week/7 days}) \\ & \times (14.6\% \text{ lower risk/100 mg omega-3 FA})] \\ & - \{[(\text{hair Hg change/fish meal}) \\ & \times (\text{no. meals/week})] - (0.51 \text{ ppm hair Hg})\} \\ & \times (23\% \text{ higher risk/1 ppm hair Hg}) \end{aligned}$$

$$\begin{aligned} \text{Net risk/benefit for infant VRM} = & \\ & [(\text{omega-3 FA mg/meal}) \times (\text{no. meals/week}) \\ & \times (1 \text{ week/7 days}) \\ & \times (2 \text{ VRM points/100 mg omega-3 FA})] \\ & - [(\text{hair Hg change per fish meal}) \\ & \times (\text{no. meals/week}) \\ & \times (7.5 \text{ VRM points/1 ppm hair Hg})] \end{aligned}$$

Species that yield a positive result from these equations have a net benefit, whereas a result < 1 signifies an increased risk.

The omega-3 FA CHD benefit may saturate above a certain daily dose, estimated by Mozaffarian and Rimm (2006) at 250 mg/day. However, as described above, this may be an artificial saturation due to the increasing effect of MeHg at higher fish ingestion rates and the evidence of no saturation of benefits in some studies. Therefore, this analysis does not include a saturation function for the omega-3 FA benefit.

These risk/benefit equations contain exposure components based on the number of fish meals eaten per week and the MeHg and omega-3 FA content of the fish. These contents are species specific. Table 2 provides estimates for these fish constituents based on data from the U.S. Department of Agriculture (USDA 2005) for omega-3 FA (DHA + EPA) and from the Food and Drug Administration (FDA 2006) for MeHg. There are a variety of other sources for omega-3 FA content of fish (e.g., American Heart Association 2008; Mozaffarian and Rimm 2006), but these other sources tend to either use the USDA data or to report very similar results. More extensive data for both omega-3 FA and MeHg content of fish (numbers and varieties of fish sampled, seasonal and source variation) are needed to improve confidence and understand variability in this key input data. The list of fish chosen for analysis is based on those commonly available in Connecticut markets and

for which MeHg and omega-3 FA data are available. This approach can be applied to any additional species as long as the MeHg and fish oil content of these species are known.

We converted the MeHg fish concentration (micrograms per gram) to a hair MeHg concentration (micrograms per gram) via a one-compartment model that relates MeHg intake to hair mercury as used in the U.S. EPA's RfD for mercury (Ginsberg and Toal 2000; Rice et al. 2003). The assumed meal size was 6 oz (170 g) of fish, with other parameters used in the model as reported previously (e.g., 95% absorption of MeHg in the gastrointestinal tract; MeHg elimination rate equals 1.4% of body burden per day). We chose a 6-oz meal size to match the recommendation used in the joint FDA/U.S. EPA seafood consumption advisory of two meals per week equivalent to 12 oz of fish (U.S. EPA 2004). Use of the Guallar et al. (2002) dose response required conversion of toenail mercury biomonitoring data to hair mercury. We accomplished this with the factor recently developed by Ohno et al. (2007) (hair mercury in micrograms per gram =  $2.44 \times$  toenail mercury in micrograms per gram). They based this factor on the regression slope between hair and toenail mercury in 57 women, which yielded a strong correlation with only a modest degree of variability and few outliers. The strength of the correlation between hair and toenail mercury provides support for the use of toenail mercury as biomarker in the Guallar et al. (2002) study.

## Results

Figures 1–3 show the integrated risk/benefit analysis for seafood consumption by end point and species. Figure 1 shows estimates of the influence of MeHg on neurodevelopment at 6 months of age (VRM score) in the 16 fish species chosen for analysis. We modeled these effects based on long-term consumption of one meal per week for several months, enough time to reach steady-state blood and hair concentrations of MeHg. We assumed that the omega-3 FA benefit requires consistent exposure over time and that no other fish were consumed other than the one meal per week of the indicated species.

Figure 1 shows a range of effects, from a large negative effect for swordfish and shark to modest positive effects for trout, farmed salmon, and herring. The rest of the species are in an intermediate zone of rather small net effect in the positive or negative direction. Canned tuna, both light (primarily skipjack) and white (primarily albacore), show negative deflections, with white tuna predicted to have a 3.7-fold larger negative impact than light tuna. Consumption of more than one meal per week on a regular basis would accentuate the pattern shown in Figure 1 because there are no

**Table 2.** Estimated omega-3 FA and MeHg levels in commonly eaten fish.

Fish species	Omega-3 <sup>a</sup> (mg/6 oz)	MeHg <sup>b</sup> (μg/g)
Cod, Atlantic	269	0.11
Flounder/sole	852	0.05
Halibut	1,398	0.26
Herring, Atlantic	3,424	0.04
Lobster	1,129	0.24
Pollack	922	0.06
Salmon, Atlantic, farmed	3,658	0.014
Sea bass	1,295	0.27
Shark	1,170	0.99
Shrimp	536	0.01
Swordfish	1,392	0.97
Tilapia	240	0.01
Trout	1,744	0.03
Tuna, canned, light	425	0.12
Tuna, canned, white	1,462	0.35
Tuna, fresh, yellowfin	474	0.325

<sup>a</sup>Omega-3 FA represents the sum of EPA and DHA. Shark data from Mozaffarian and Rimm (2006); other data from USDA (2005). <sup>b</sup>MeHg data from FDA (2006); data for salmon reported as fresh/frozen and not distinguished according to source.

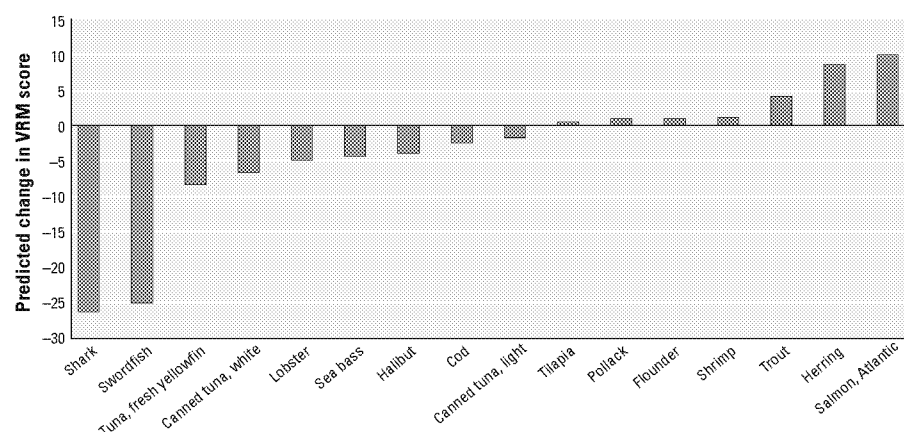
known thresholds or saturation limits to the MeHg decrement or omega-3 FA benefit for neurodevelopment. This means that the intermediate species in the center of Figure 1 would have larger positive and negative deflections the more meals ingested per week. This is a possible concern for species such as fresh tuna (tuna steak), canned white tuna, lobster, and sea bass. In contrast, the marginal benefit of species such as tilapia, pollack, flounder, and shrimp may increase with greater meal frequency. The negative impacts of swordfish and shark and the beneficial effects of trout, farmed salmon, and herring would also be magnified as consumption of these species goes up. However, the presence of other contaminants in species such as farm-raised salmon (Hites et al. 2004) needs to be considered when recommending frequent fish consumption.

Figure 2 shows the net benefit or risk of fish ingestion on CHD mortality and MI. Not surprisingly, the pattern across species is similar to that shown in Figure 1 because, in our framework, the net benefit or risk is contingent upon the ratio of omega-3 FA to MeHg in the fish, which does not change when analyzing different end points. However, the results in Figure 2 suggest that the risk/benefit ratio is more in the benefit direction for CHD mortality compared with VRM score. This can be seen by the number of species with positive deflections in Figure 2 (13) compared with Figure 1 (7), with such commonly eaten foods as canned tuna and cod having a beneficial influence on the cardiovascular end point but negative influence on the neurodevelopmental end point. One reason for the greater benefit of fish consumption on this end point is the MeHg threshold built into this equation. Species in the central portion of Figure 1 have low to intermediate levels of both MeHg and omega-3 FA; these species are at or below the MeHg effect threshold, thus allowing their modest level of omega-3 FA to be the primary influence. The underlying slope factors are also more favorable for a net benefit in the case of cardiovascular risk. However, we estimated a substantial risk for those whose fish ingestion consists of swordfish or shark; the negative deflection reflects an approximately 50% worsening of the relative risk for MI. In contrast, we estimated an approximately 75% improvement for salmon and herring. These effects are magnified for species on either end of the spectrum when simulating two 6-oz meals per week for each species (Figure 3). Intermediate species show little change at two meals per week, an indication that they have surpassed the MeHg toxicity threshold with this increase in consumption, and this prevents a further benefit from more omega-3 FA intake. The species-specific risk/benefit pattern did not change when evaluating four meals per week (data not shown).

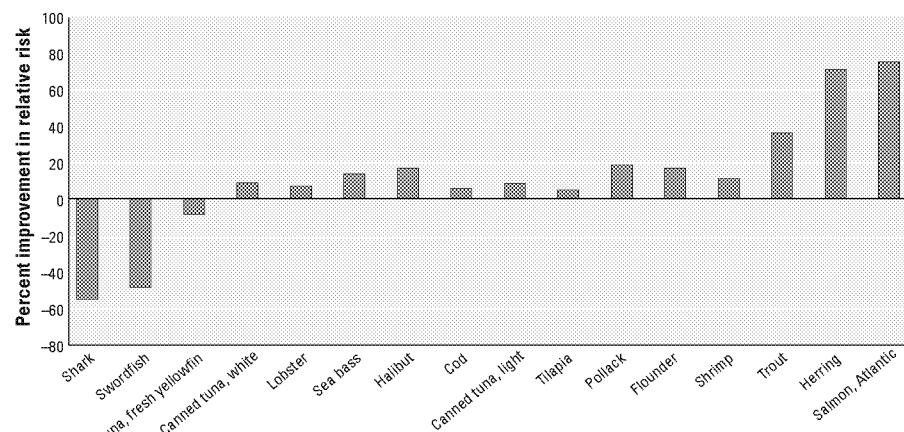
The present risk/benefit analysis allows us to tentatively classify these species into categories of fish consumption. Table 3 presents four consumption categories to illustrate how this analytical framework can be used to guide advisories. The species-specific risk/benefit rankings were sufficiently different across end points to yield slightly different advice for

those in the neurodevelopmental risk group versus the cardiovascular risk group.

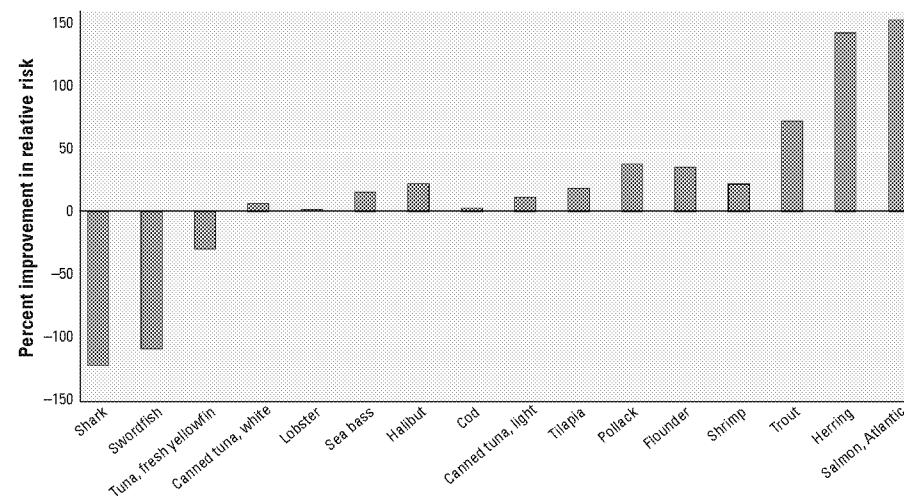
We have tentatively created an unlimited category because, for the end points and constituents analyzed, increasing consumption of certain fish was associated with an increasing benefit. A caveat is the evidence for a saturation of the omega-3 FA cardiovascular benefit



**Figure 1.** Estimated net effect of MeHg and fish oils on neurodevelopment at 6 months of age, one 6-oz fish meal per week.



**Figure 2.** Estimated net effect of MeHg and fish oils on cardiovascular risk, one 6-oz fish meal per week.



**Figure 3.** Estimated net effect of MeHg and fish oils on cardiovascular risk, two 6-oz fish meals per week.

> 250 mg/day (Mozaffarian and Rimm 2006), but as described above, saturation of the benefit is speculative for cardiovascular risk and is not evident in the limited analyses available for neurodevelopmental risk (Hibbeln et al. 2007; Oken et al. 2005, 2008). However, because of persistent organochlorine contaminants in certain species such as farmed salmon, one must consider consumption limits based on cancer risk or other end points (Foran et al. 2005). Data for such contaminants should be analyzed to make sure that unlimited consumption of these species is appropriate.

Only a few species are in the twice-per-week consumption category, which is the general seafood advice from the FDA (2004). This is because the largest category is unlimited consumption, containing seven species for the neurodevelopmental risk group. Unlimited consumption is taken to mean one 6-oz meal per day. Figures 1–3 show these species to be associated with net beneficial effects, regardless of the number of meals per week. Fish were placed in this category if they have a net beneficial effect and also if the RfD for mercury (0.1 µg/kg/day) is not exceeded from daily fish ingestion. For the neurodevelopmental risk group, cod and canned light tuna have a slight negative deflection in Figure 1, but were placed in the twice weekly category because when eaten at this frequency they provide less MeHg than the neurodevelopmental RfD and are unlikely to be a significant risk, given the various uncertainties and the fact that there are other nutrients in fish. We included five species in the once weekly category (canned white tuna, tuna steak, halibut, sea bass, lobster). Although we estimated them to yield a net risk at one meal per week (Figure 1), they are also at or below the neurodevelopmental RfD at this frequency. Swordfish and shark have considerably more MeHg, and are estimated to have a much greater net risk and so are in the “do not eat” category. Of the 16 species analyzed, none fit

into a one meal per month category, although that may be appropriate for other fish.

For the cardiovascular risk group, unlimited consumption appears to be appropriate for nine species, and potentially several more (Figures 2, 3). However, we downgraded canned white tuna, halibut, sea bass, and lobster to two meals per month because of concerns for neurologic effects. There is no MeHg RfD relevant for the general population, but a number of states have used a 3-fold higher target dose (0.3 µg/kg/day) given the likely differences in sensitivity for neurologic effects between early life and adults (McCann 2005); this target dose is the same as the IRIS RfD for inorganic mercury salts (U.S. EPA 1995). Thus, we placed species in the twice weekly category to keep MeHg exposure below the target dose for the general public to prevent neurologic effects. Tuna steak was placed in the once weekly category to limit the risk as estimated in Figures 2 and 3, which is very small at once per week. We estimated swordfish and shark to have a substantial net risk, even at one meal per month; thus, they are in the “do not eat” category.

## Discussion

This analysis presents a first attempt at a model that can be refined in the future as more data become available on cardiovascular and neurodevelopmental risks of MeHg, and the health benefits of consuming fish and fish oils. Although we acknowledge that there are limitations in the data used to derive this model, there appears to be an obvious utility to this approach. Public health officials need to weigh the positive and negative aspects of particular fish species when crafting advisories, but to date, there is no well-accepted, objective method to do this. Using this model, we have placed species commonly available in Connecticut into four consumption categories to illustrate the potential utility of the model. These consumption rates can be used as a point of comparison with rates

being recommended by the FDA, the U.S. EPA, and various medical and public health authorities, after recognizing the limitations of the present analysis.

We considered the influence of fish consumption on end points that are among the most sensitive for the beneficial effects of omega-3 FA and the toxicity of MeHg. The analysis addresses two completely different groups (adults and the fetus) and encompasses 16 different species, yet it is simplistic in only assessing two factors regarding fish ingestion that may influence these end points. Other nutrients such as protein, selenium, iron, and iodide and other contaminants such as polychlorinated biphenyls, persistent pesticides, and dioxins (Bocio et al. 2007; Hites et al. 2004) may also be contained in these species. We chose constituents (omega-3 FA, MeHg) that have a mechanistic basis for influencing cardiovascular and neurodevelopmental outcomes and have actually been shown to do so in a variety of animal and human studies (Cohen et al. 2005a; IOM 2006; Mozaffarian and Rimm 2006). However, the potential importance of other constituents and end points creates uncertainty regarding the overall health implications of fish consumption.

It is important to recognize that fish ingestion has shown a beneficial effect on neurodevelopmental and cardiac outcomes in a number of studies (Daniels et al. 2004; Hibbeln et al. 2007; IOM 2006; Mozaffarian and Rimm 2006; Oken et al. 2005). Therefore, an important public health message is that fish are a key dietary component. However, this can also be incorrectly interpreted to mean that, despite MeHg contamination, fish ingestion is a positive influence and consumption limits are unnecessary (Hibbeln et al. 2007). Results from general population studies are likely a reflection of the types of fish eaten. If the studied population ingested more beneficial fish (Figures 1–3), this can create the appearance of a generalizable positive association in the absence of information on the actual species consumed. However, the present analysis and those of others (Guallar et al. 2002; Mahaffey et al. 2007; Oken et al. 2005, 2008; Stern 2007) point out the importance of looking at individual species because the risk/benefit ratio may vary considerably across species. A species-by-species approach to consumption advisories is particularly meaningful given that many people have favorite fish they eat most often. The goal of the species-specific approach is to encourage people to eat from a variety of fish, focusing on the most beneficial species.

Our analysis is supportive of the federal advisory (FDA 2004; U.S. EPA 2004) in showing that certain species should not be eaten by women of childbearing age (swordfish, shark; federal advisory also lists king mackerel and tilefish). In addition, we provide

**Table 3.** Tentative fish consumption categories for the 16 species analyzed in the present risk/benefit assessment (based on 6-oz meal size).

Risk group	Consumption category	Fish species
Neurodevelopmental <sup>a</sup>	Unlimited (pending evaluation of other contaminants) <sup>b</sup>	Tilapia, pollack, flounder, shrimp, trout, herring, salmon
	Twice per week	Canned light tuna, cod
	Once per week	Canned white tuna, tuna steak, halibut, sea bass, lobster
	Do not eat	Swordfish, shark
Cardiovascular <sup>c</sup>	Unlimited (pending other contaminants) <sup>c</sup>	Tilapia, pollack, flounder, shrimp, trout, herring, salmon, canned light tuna, cod
	Twice per week	Canned white tuna, halibut, sea bass, lobster
	Once per week	Tuna steak
	Do not eat	Swordfish, shark

<sup>a</sup>Pregnant women, women of childbearing age, nursing mothers, young children. <sup>b</sup>Unlimited taken to mean daily consumption. <sup>c</sup>General adult population.

risk/benefit support for separate two meal and one meal per week categories. The federal advisory generally recommends two 6-oz seafood meals per week but does specifically limit canned white tuna to one meal per week. The present analysis goes beyond that to list other species that are candidates for the once weekly category. Further, we provide a list of species that may potentially be safely consumed at greater than the meal frequency recommended by the federal advisory, on the basis of neurodevelopmental and cardiovascular risks, without taking into account other contaminants and end points of potential concern.

This assignment of consumption advice for individual species is tentative given the limitations inherent in the present analysis. The dose-response relationships for the risks and benefits of these components (Table 1) are supported by the available data but do contain uncertainties. The omega-3 FA benefit for acute cardiovascular risk has been documented in numerous epidemiology studies, and the dose response shown in Table 1 is a synthesis of 20 different studies (Mozaffarian and Rimm 2006). However, many of these studies involved fish consumption rather than omega-3 FA supplementation; therefore, the effect of a single nutrient (omega-3 FA) is uncertain, given that other nutrients in fish may have contributed to the observed benefit. Although this remains an uncertainty, omega-3 FA is a well-established benefit for cardiovascular risk and is the main fish nutrient for which dose-response relationships have been reported. Therefore, this is the most feasible approach at the current time. This is not an area of possible underestimation of fish benefits. The benefits attributed to omega-3 FA in the fish consumption studies come from all nutrients, not just the fish oils, because we made no attempt to separate out these other benefits. From this perspective, the omega-3 FA dose-response functions developed in the present analysis will tend to capture the overall benefit of fish consumption, except for the limited extent to which studies of fish oil supplements contribute to the supporting database.

MeHg effects on heart function and blood vessels have been reported in animal studies, cell cultures, and two large epidemiology studies (ATSDR 1999; Guallar et al. 2002; Mazerik et al. 2007; Rissanen et al. 2000; Salonen et al. 1995, 2000; Virtanen et al. 2005). This includes a series of four reports from a group of men in eastern Finland whose diet was enriched in fish that are low in omega-3 FA and relatively high in MeHg (Guallar et al. 2002; Rissanen et al. 2000; Salonen et al. 1995; Virtanen et al. 2005). This cohort provides a good opportunity to document an MeHg effect without much compensation by dietary omega-3 FA. Guallar

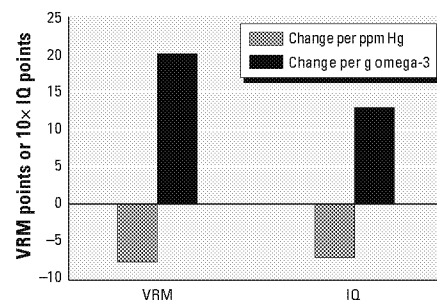
et al. (2002) studied a different population of men from across Europe and Israel in whom MeHg exposure varied substantially based on country of residence and sources of fish intake. The association of MeHg with increasing cardiovascular risk was evident even without correction for DHA exposure, but the association was strengthened once DHA was considered. These findings provided a reasonable dose response for the present study (Table 1) despite the fact that several studies have not shown such an association with inorganic mercury or MeHg (Ahlqvist et al. 1999; Hallgren et al. 2001; Yoshizawa et al. 2002). One of these was primarily a study of occupational exposure to elemental mercury from dental amalgam (Yoshizawa et al. 2002), another was in Swedish women rather than men and also appears to have amalgam as a primary source of mercury (Ahlqvist et al. 1999), and the more recent Swedish study had too few subjects with elevated mercury exposure (Hallgren et al. 2001). Therefore, these studies are not substantial counterweights to the positive findings in European men described above. However, these positive findings are limited in coming from only two data sets, eastern Finland and the Guallar et al. (2002) results, with a useful dose-response analysis available only in the latter case. It is possible that reanalysis of the eastern Finland data could further support this dose response, because Salonen et al. (1995) found an odds ratio (2.0) for elevated hair mercury ( $\geq 2$  ppm) similar to that found by Guallar et al. (2002). Further exploration of MeHg effects on cardiovascular risk is critical for establishing fish consumption advice that is adequately protective for this end point.

Regarding the neurodevelopmental dose response shown in Table 1, both the omega-3 FA benefit and MeHg risk factors were derived by Oken et al. (2005) from an analysis of VRM scores in 6-month-old children. The group in that study which most clearly showed the MeHg effect was small (high hair mercury, low fish intake;  $n = 12$ ). However, other data corroborate this dose response. Figure 4 shows our comparison of data from Oken et al. (2005) with dose-response factors for a related end point, IQ, as synthesized from several studies (Cohen et al. 2005a, 2005b, 2005c). The ratio of omega-3 FA benefits to MeHg risks is similar across these studies, with the dose response for IQ somewhat less in the benefit direction than the one we used based on VRM. Therefore, it is unlikely that we are underestimating the net fish benefit on neurodevelopment by using the Oken et al. (2005) analysis as our basis. A recent follow-up with this group of mother-child pairs found neurodevelopmental evidence of fish ingestion benefits and MeHg risks extending out to 3 years of age (Oken

et al. 2008). There was evidence of a beneficial influence of omega-3 FAs on these outcomes, but this did not attain statistical significance, possibly due to the uncertainties in calculating omega-3 FA intake from diaries of fish consumption. The role of fish oils and other fish nutrients in assisting brain development needs to be a continuing research focus.

Our analysis is limited in that we assessed each fish species in isolation from consumption of any other fish. People generally eat a variety of fish, although some may have a strong preference for one particular species. A robust analysis of food dietary patterns can be used to assess what fish the U.S. population eats (Carrington and Bolger 2002) and how this influences the risk/benefit equation across average or upper-bound consumers. Other variabilities not expressed in our analysis are important to explore and build into more refined analyses: the variability in fish concentrations in omega-3 FA and MeHg, the variability in the toxicokinetics of MeHg, and the variability in response functions for omega-3 FA and MeHg. Although the present analysis does not address population risk, it provides a useful framework for analyzing species-specific risks and benefits that need to be considered when deriving fish advisories. This is critical because a number of states, including Connecticut, are evaluating ways to highlight beneficial fish and discourage consumption of the riskier species.

Several other analyses of the risks and benefits of fish consumption have been published that range from purely qualitative to more quantitative estimates of net risk or benefit. The IOM (2006) provided a qualitative summary and recommended that fish be included in the diet but within federal consumption guidelines. Adults at risk for cardiovascular disease are recommended to eat two 3-oz meals per week as a preventative measure. Above this consumption rate, the IOM recommends diversifying the fish intake to minimize the chance of excessive MeHg exposure from particular fish sources. Mozaffarian and



**Figure 4.** Comparison of estimated effect sizes for MeHg and omega-3 FAs on IQ (Cohen et al. 2005a) and VRM (Oken et al. 2005). Scale for IQ points is multiplied by 10 to adjust size of bars for easy viewing relative to VRM score.

Rimm (2006) performed a more quantitative assessment of dose–response relationships for fish oil benefits on cardiovascular outcomes, but they did not provide a quantitative assessment of MeHg risks. Their risk/benefit assessment was mostly qualitative and concluded that consuming one to two servings of fish per week is beneficial in adults and in women of childbearing age, although the latter group should be wary of a few high MeHg species. In an analysis of seafood available in New Jersey markets, Burger et al. (2005) focused on cost and health considerations but not the benefits of omega-3 FA. They found that flounder was the most economical species that is low in MeHg. Cohen et al. (2005a) used quality-adjusted life years (QALYs) to put the MeHg risks to cognitive development on a common scale with fish benefits for CHD mortality, stroke prevention, and DHA benefits for neurodevelopment. Their analysis looked at how consumption patterns may shift in response to fish advisories and found a net benefit if advisories are properly followed but substantial health risks if advisories lead to unnecessary decreases in fish consumption. Ponce et al. (2000) also used QALYs to contrast MeHg neurodevelopmental risk with fish oil cardiovascular benefits. Their article had the drawback of mixing different end points and receptor types (early life and adult) into a single analysis. Domingo et al. (2007a, 2007b) provided data on omega-3 FA and contaminant levels in 14 species sampled from fish markets in Catalonia, Spain. They analyzed whether certain dietary patterns would result in contaminant intakes above tolerable daily intakes and whether omega-3 FA intakes were adequate with respect to recommendations of international heart associations. In a somewhat similar vein, Foran et al. (2005) quantitatively assessed the risks and benefits of farmed and wild salmon consumption with benefits entered into the equation as the omega-3 FA content of the meal and the risk assessed based on the cumulative cancer or noncancer risk of the contaminants (target cancer risk,  $1 \text{ in } 10^{-5}$ , target noncancer risk of unity). Their analysis found that farmed salmon should be limited, from less than one meal per week to three meals per week depending on source, to meet the World Health Organization (1998) target dose for dioxin equivalents ( $1 \text{ pg/kg/day}$ ), with farmed salmon from European sources generally on the low end of this consumption advice. These frequencies were still associated with elevated cancer risk, although they also contained substantial omega-3 FA benefit. It is important to keep in mind that trace levels of carcinogens are in many foods, so the relative risk/benefit ratio of a source such as salmon should ideally be compared against other protein sources (e.g., meat, dairy, vegetarian sources) if cancer

is a critical end point. Along these lines, the levels of dioxins found in some farmed salmon are greater than what is typically available from other protein sources. The Connecticut Department of Public Health's latest seafood advisory is for no more than one meal per week of farmed salmon on this basis.

It may be theoretically possible to obtain omega-3 FA benefits and avoid some of the contaminant issues by taking fish oil supplements. Other foods that are fortified with omega-3 FA, such as eggs and milk, can be an additional source. For example, chickens fed diets containing ground flaxseed lay eggs that are enriched in omega-3 FA ( $\sim 500 \text{ mg/egg}$ ), although most of this is in the form of  $\alpha$ -linolenic acid, which has less evidence for neurodevelopmental and cardiovascular benefits compared with fish oils (FDA 2005). Another form of omega-3 FA fortification of eggs has been developed that involves supplementation of the hen's diet with marine microalgae, a source reportedly rich in DHA ( $150 \text{ mg/egg}$ ). Publicly available test data regarding the omega-3 FA content of these supplemented foods are needed to understand their potential benefit. Another consideration is that replacement of fish with supplements or fortified eggs will not necessarily provide other nutrients that fish offer (e.g., iron, selenium, iodide). These nutrients are not being analyzed in present risk/benefit analyses but they may be part of the benefit being attributed to fish-oil ingestion. In general, nutrition authorities recommend obtaining nutrients from the whole food rather than from extracted or chemically synthesized components. Finally, omega-3 FA supplements are not regulated by the FDA, so label accuracy, quality control, and contaminant testing may be issues. Clearly, the beneficial effects of omega-3 FAs on cardiovascular and neurodevelopmental outcomes need to be further explored in relation to the overall benefits of fish consumption to refine species-specific advice and to make recommendations about the utility of fish oil supplements.

In contrast to previous risk/benefit analyses, the present study is the first to provide an integrated analysis for MeHg and omega-3 FA that uses dose–response relationships on common end points and that evaluates the net effect on a species-by-species basis. This approach and the resulting consumption categories illustrate a framework that should be helpful in establishing advisories for a wide variety of commercially available and locally caught fish, assuming that the requisite MeHg and omega-3 FA data are available. We believe this can help resolve the confusion that currently exists regarding fish consumption and yield a message that focuses on the most beneficial fish choices without eliciting fear over the dangers of MeHg. Currently, there are

numerous uncertainties regarding additional contaminants, nutrients, end points, underlying dose–response functions, and comparisons with other protein sources. These factors would require a more data intensive and complex analysis, but this is an important direction for the future (Domingo et al. 2007b; Foran et al. 2005).

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Review

# Approaches to Children's Exposure Assessment: Case Study with Diethylhexylphthalate (DEHP)

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**Abstract:** Children's exposure assessment is a key input into epidemiology studies, risk assessment and source apportionment. The goals of this article are to describe a methodology for children's exposure assessment that can be used for these purposes and to apply the methodology to source apportionment for the case study chemical, diethylhexylphthalate (DEHP). A key feature is the comparison of total (aggregate) exposure calculated via a pathways approach to that derived from a biomonitoring approach. The 4-step methodology and its results for DEHP are: (1) Prioritization of life stages and exposure pathways, with pregnancy, breast-fed infants, and toddlers the focus of the case study and pathways selected that are relevant to these groups; (2) Estimation of pathway-specific exposures by life stage wherein diet was found to be the largest contributor for pregnant women, breast milk and mouthing behavior for the nursing infant and diet, house dust, and mouthing for toddlers; (3) Comparison of aggregate exposure by pathways vs biomonitoring-based approaches wherein good concordance was found for toddlers and pregnant women providing confidence in the exposure assessment; (4) Source apportionment in which DEHP presence in foods, children's products, consumer products and the built environment are discussed with respect to early life mouthing, house dust and dietary exposure. A potential fifth step of the method involves the calculation of exposure doses for risk assessment which is described but outside the scope for the current case study. In summary, the methodology has been used to synthesize the available information to identify key sources of early life exposure to DEHP.

**Keywords:** children; pregnancy; breast milk; house dust; contaminants; exposure assessment; plasticizer; phthalate; DEHP

## 1. Introduction

Exposure assessment is a critical aspect of any chemical evaluation. In some cases, evidence of exposure precedes a complete understanding of chemical effects and so can be the impetus for new toxicology studies or the exploration of exposure sources. For example, the biomonitoring evidence of widespread and increasing human exposure to polybrominated diphenyl ethers (PBDEs) in the 1990s and early 2000s spurred new toxicology and exposure research [1]. This need is especially great when the exposure profile of the chemical intersects with children. Children are a high priority because their toxicokinetic handling and toxicodynamic response to chemicals is often different than the remainder of the population [2,3]. Further, children's physiological and behavioral factors can make them the most highly exposed sector of the population for chemicals that have widespread distribution. This results from their higher metabolic demands leading to increased food, water and air intake per body weight, as well as behaviors that increase contact with contaminants in soil, house dust and products (e.g., crawling, mouthing behavior) [4,5].

This manuscript's goals are to [1] describe current concepts in children's exposure assessment, organizing this information into a broadly applicable methodology; and [2] illustrate the method with a case study for the plasticizer diethylhexylphthalate (DEHP). This chemical was chosen because of its high frequency of detection in children's environments, as well as having an extensive database that includes biomonitoring, indoor air, house dust, food, and consumer product data [6–11]. These exposures may be particularly relevant for early life stages because of the known endocrine disrupting and developmental effects of this plasticizer [12,13]. DEHP's endocrine disrupting effect has an early life window of heightened vulnerability which can alter male in utero development and have long-term implications for reproductive health [14]. Early life exposures are also associated with an increased tendency for respiratory allergy in children [15]. DEHP exposure assessment is critical to understanding the sources and levels of exposure, as well as the options for decreasing exposure in children [16].

## 2. Types of Exposure Assessment for Children

Risk assessments have traditionally captured children's exposure through the use of higher contact rates with soil, water and food for the period of childhood for which these rates are relevant. For example, the standard adjustment for children's soil ingestion rate over the first six years of life is approximately 10 times greater than that assumed for adults on a kg body weight basis [17,18]. However, the purpose of children's exposure assessment is more than deriving adjustment factors for risk assessment. The following list presents various uses of a children's exposure assessment including and in addition to risk assessment. It is important to consider the use and purpose of the exposure information in the scoping stage as this may affect the approach and methodology:

I. Exposure assessment for risk assessment: objective is to develop a dose in mg/kg/d (or equivalent) for a specific exposure scenario that involves one or more types of contaminated media. The dose can be specific to a given lifestage such as children, or can include various age groups and be cumulated across the entire lifespan as the average daily dose [19]. This calculation can involve three different approaches: (a) lifetime average daily dose, typically what is used for carcinogenic risk; (b) lifetime average daily dose cumulated over different lifestages; for mutagenic carcinogens this incorporates age dependent adjustment factors (ADAFs) to modify cancer potency [20] and age-specific exposure rates such that certain periods in early life can make a greater contribution to lifetime risk than other periods; (c) average daily dose cumulated over the exposure period being analyzed (e.g., 6 years of childhood or chronic 30 year period as adults). This latter method is often the way exposure is considered for non-cancer risk assessment.

II. Exposure assessment for source apportionment: in some cases calculation of risk may be uncertain or premature due to questions regarding the toxicology database, but there is still an interest in identifying the highest exposure pathways to consider prudent mitigation options. This type of exposure assessment focuses less on the overall dose but rather on identifying key pathways of exposure and the source of chemical to each pathway.

III. Exposure assessment for epidemiology studies: epidemiology studies typically don't need a dose to correlate exposure with outcome. They need to place individuals into categories of exposure based upon some type of measure (e.g., exposure survey, environmental measurement, biomonitoring) that indicates whether the individual has received a low, medium or high level of exposure [21]. While an estimate of dose per body weight per day can be helpful, it is not required in epidemiology to the same degree as in risk assessment.

IV. Exposure assessment to capture status and trends: measurement of exposure over time can reveal important temporal trends that can be related back to changes in regulations, manufacturing practices and the consumer marketplace. The classic example is the temporal correlation between removal of lead from gasoline and the decline in childhood blood lead [22]. Measurement across different sectors of the population can identify how lifestage, gender, occupation, ethnicity or behavioral factors may affect exposure, while comparison of exposure across different countries can show geographical differences. These descriptive statistics can point out key exposure vulnerabilities.

Status and trends assessments are commonly based upon biomonitoring data as opposed to calculations of exposure dose.

V. Exposure assessment to prioritize chemicals, products, sources. The previous types of exposure assessments can be applied to the prioritization of chemicals based upon such considerations as frequency of detection in biomonitoring studies, media where detections occur and concentrations found, whether dose estimates approach or exceed risk-based targets, and trends in exposure over time. The following state governments have prioritized chemicals of concern to children's health if they meet their definition of toxicity and have evidence of exposure that can include presence in house dust, indoor air, consumer products or detection in biomonitoring studies: Maine [23], Washington [24], Vermont [25] and California [26].

A further consideration is whether one is primarily interested in a particular exposure pathway or in aggregate exposure, the sum across all pathways possible for a given receptor. Aggregate exposure assessment is valuable to assess the relative importance of various pathways, with the estimate of total daily dose of potential use in a risk assessment or within epidemiology studies. Ideally the estimate of aggregate dose can be corroborated between a biomonitoring-based and a pathways-based analysis. In addition to aggregate exposures considered in this analysis, cumulative exposure can also be considered to include exposures across multiple chemicals (for example similarly-acting chemicals such as dioxin congeners, phthalates and within certain classes of pesticides) and non-chemical stressors (for example, lack of access to health care).

### 3. Children's Exposure Assessment Approach

The development of an exposure assessment that can meet the various purposes described above is aided by following a method that ensures that key developmental windows and relevant exposure pathways are considered. The United States Environmental Protection Agency (USEPA) developed a children's exposure and risk assessment framework in 2006 and has drafted updated human exposure guidelines [27,28]. A simplified 4 step approach that can be used to evaluate the existing exposure information for a well-studied chemical is outlined below and then applied to DEHP as an illustrative case study.

Four Step Approach for Assessing Children's Exposure to Environmental Chemicals:

1. Prioritize exposure pathways and age groups for quantitative analysis.
2. Estimate pathway-specific and aggregate exposure.
3. Compare estimate of aggregate exposure from pathways analysis to dose estimates from biomonitoring studies to assess whether results from these different approaches are consistent and thus lead to greater confidence in the overall assessment. Estimates of aggregate exposure can also be compared to toxicity values in a risk assessment as mentioned below.
4. Determine which are the quantitatively most significant exposure pathways and explore which sources of chemical are of importance to these pathways.

A 5th phase is added if conducting a risk assessment, that is to aggregate exposures as doses in a manner compatible with calculation of health risk for cancer (multiplied by the cancer slope factor) or non-cancer (divided by the reference dose (RfD)) depending upon the health outcomes of concern. While central tendency estimates of exposure may suffice for phases 1–4, the risk assessment calculations may also consider the full range of exposures possible to understand whether any members of a particular age group may experience an elevated risk. The scenario and toxicity endpoints being analyzed may also dictate that exposures be calculated for short-term peak exposures rather than a long-term average exposure. If long-term exposure is evaluated, the cumulative dose over different early life stages (e.g., at birth from transplacental, breastfeeding, toddler, school-age, adolescent) may need to be estimated.

#### 4. Using the Children's Exposure Assessment Methodology for DEHP

DEHP is a general purpose high molecular weight phthalate plasticizer used to make polyvinylchloride (PVC) plastic flexible where its content is typically on the order of 30% [29]. PVC polymers have a wide range of uses including in building materials such as wiring and cable coatings, flooring and wall paper, and in consumer products ranging from shoes and shower curtains to vinyl table cloths, carpet backing and furniture upholstery [30,31]. DEHP is also used in a variety of non-PVC applications such as an ingredient in sealants, lacquer and paint. Its use in medical tubing, intravenous bags and similar medical devices has been recognized as an important source of exposure to premature infants and children needing hospitalization, with FDA recommending that alternatives be considered [32,33]. On an acute basis this exposure source can be larger than other pathways analyzed [34,35] and it has been associated with health effects in pediatric patients [32]. However, given that this is a specialized exposure setting that is not widespread across the community, it is not explicitly considered in the current case example.

Of the purposes described above, chemical prioritization, risk assessment, status and trends, and source apportionment, we select the latter, evaluation of pathways and sources, for this case study analysis. DEHP has already been prioritized as having the highest toxicity/exposure rank amongst house dust contaminants assessed in a study focusing on data from France [8]. Biomonitoring studies show that of the phthalate metabolites analyzed those stemming from DEHP were among the highest across the US population including children [36]. An analysis across the Center for Disease Control (CDC) biomonitoring database found that DEHP was one of only a few analytes whose levels indicated a potential exceedance of a health benchmark [37]. In 2014 USEPA prioritized DEHP for further assessment by adding it to its list of Toxic Substances Control Act (TSCA) work plan chemicals [38]. Driving these concerns are the endocrine disruptive effects demonstrated for phthalates in general and DEHP in particular in early life stages [16]. A detailed exposure assessment of DEHP in children is warranted given its widespread detection across the population with indications of higher exposure in young children [6,36]. This case example provides an identification of key exposure pathways, an evaluation of aggregate exposure and sources of exposure to children. This information can help inform strategies to mitigate children's exposures to DEHP if risks are found to be high or if replacement chemicals are identified which have a more favorable toxicology profile.

##### *4.1. Phase 1. Scoping Developmental Life Stages and Exposure Pathways for Inclusion in a Detailed Assessment*

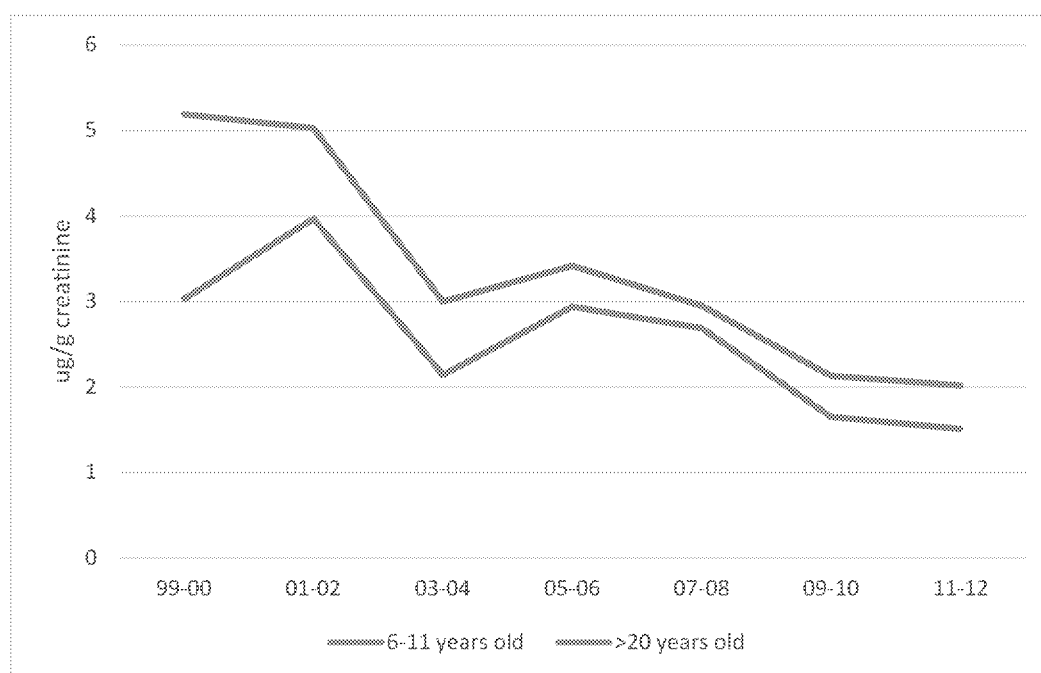
The first goal of exposure assessment is to determine the key pathways whereby sensitive early lifestages may contact the subject chemical. This is informed by a series of questions regarding the potential for children to receive DEHP exposure:

- Is DEHP contained in products designed for children or that children frequently use?
  - For the most part the answer to this question is no, or at least not as much as in the past. DEHP presence in toys and childcare articles was limited in the US by federal legislation [39] to a nominal level <0.1%. Exempt from this limit is children's footwear and most forms of clothing. It is also possible that toys and articles produced prior to the setting of limits are still found in homes.
- Is the chemical detected in media children frequently contact?
  - Yes, DEHP is present in consumer products that children can contact such as flooring, shower curtains, upholstery and car interiors. House dust studies routinely find it to be the highest phthalate in this medium with levels also detectable in indoor air; however, indoor air levels of DEHP are typically quite low compared to lower molecular weight phthalates.
  - DEHP is not tightly bound to plastic and so can be released via physical contact (e.g., a child mouthing a DEHP containing object; wear and tear on flooring and shoes). Another important release mechanism is volatilization. In spite of its low volatility, the high surface area of many PVC items around the home leads to offgassing and accumulation in house

dust [40]. Because of DEHP's low volatility, it will tend to partition into house dust rather than be found as a gas in indoor air. Thus, house dust is the primary storage compartment for DEHP that has been released from the built environment and consumer products [40].

- Is DEHP contained in foods children can be expected to eat? Is it present in breast milk or formula?
  - Yes, DEHP was detected in 70% of the 78 breast milk samples reported in a 2011 study from Germany [41] and in over 80% of samples in a 2015 study in Korea [42]. In the German study, DEHP metabolites were found less frequently than parent compound in breast milk (monoethylhexylphthalate (MEHP) 58%, oxidized metabolites 0%). Similarly, a US study of lactating women found less than 10% of milk samples were positive for DEHP oxidized metabolites but parent compound and MEHP were not analyzed in that study [43]. DEHP has been detected with high frequency in infant formula with overall levels comparable to breast milk [41]. DEHP is a common contaminant of the human diet with analysis of food items in Belgium finding that concentrations of DEHP were typically more than an order of magnitude higher than the other phthalates [11]. DEHP's contamination of foods may stem from its use in food packaging such as metal cans, plastic wrap and storage containers. DEHP can leach from such packaging and enter the food product itself [44]. Further, certain foods may contact DEHP during production or storage prior to consumer purchase.
- Is DEHP a common drinking water contaminant?
  - Yes but at low levels. Analysis of drinking water supplies in Portugal found sporadic detection of DEHP with all detections below 1 ug/L [45]. In Mexico City DEHP was among the most commonly detected contaminants with concentrations from groundwater supplies ranging up to 0.23 ug/L and surface waters ranging up to 2.3 ug/L. The higher concentration in surface water is due to DEHP presence in aqueous discharges into rivers which are also used for drinking water [46]. Bottled water was found to contain DEHP up to 1.7 ug/L, with leaching from the plastic bottle possibly contributing to its detection [47]. Data for DEHP in public drinking water in the United States, as summarized by a USEPA six year review of public supply data, found 1.7% of supplies with an exceedance of the DEHP maximum contaminant limit (6 ug/L) and the central tendency of detected concentrations between 1 and 2 ug/L [48]. These detections were in studies that considered artefactual contamination of samples from phthalates present in sampling devices and labware but often these sources can't be completely ruled out when there are trace detections.
- Has the contaminant been detected in biomonitoring studies? Is there direct biomonitoring evidence in children?
  - Yes, DEHP is detected in urine as hydrolyzed and oxidized metabolites. These biomarkers have relatively short half-lives and so the detected level reflects recent intake. DEHP is present in food, tap water and a variety of consumer products suggesting its intake may be fairly consistent from one day to the next which would make the urinary biomarker a reasonable indicator of long-term average intake. However, repeat sampling from the same individuals indicates a relatively weak within subject correlation (interclass correlation coefficient (ICC) of 0.1 to 0.3) [49]. This source of variability will tend to weaken associations in epidemiology studies. However, statistics for DEHP urinary metabolites from large population studies such as the CDC biomonitoring dataset are useful to depict the frequency and range of concentrations in the general population. DEHP metabolites are commonly found in urine at a frequency approaching 100% [43]. The CDC dataset indicates that 6–11 years old children consistently have higher DEHP exposure than adults and that over time the trend has been for decreasing exposure (Figure 1). The CDC dataset does not have information for younger children but as summarized in a later section, limited data for younger children are available elsewhere.

The result of this pathways scoping assessment is that children's exposure to DEHP can occur via multiple routes including diet, drinking water, consumer products and the indoor environment (e.g., house dust, indoor air). Biomonitoring data suggest that concentrations in older children and adults have been decreasing over the past 12 years, with this trend compatible with the replacement of DEHP with other plasticizers during this time. However, DEHP exposure still appears to be common with the relative importance of the various pathways in need of definition. It is important to note that the DEHP case study is very data rich, and that in some cases, the life stage based exposure assessment will be undertaken with less information; this is an acceptable practice as long as the limitations of available data are clearly defined.



**Figure 1.** Temporal Trend in MEHP Urinary Concentrations in Children and Adults. Data from CDC Biomonitoring Report [50].

#### 4.1.1. Target Life Stages

Age windows for exposure assessment are selected based upon changing patterns of exposure and the potential for toxicokinetic and toxicodynamic windows of vulnerability that may be the basis for evaluation of a certain life stage in a risk assessment. USEPA's guidance on selecting age windows is a useful resource for scoping the exposure assessment [51]. The document recommends age groups based on an understanding of differences in behavior and physiology that may impact exposures in children of various ages; a consistent set of early-life age groups, supported by an underlying scientific rationale, increases the consistency and comparability across exposure assessments.

DEHP is an endocrine disruptor having a specific effect on male development in utero, being capable of causing testicular dysgenesis syndrome. As demonstrated in rats this syndrome can include reduced anogenital distance in males at birth along with the potential for hypospadias (penile birth defect) and reduced sperm count at sexual maturity [52]. Evidence that this effect occurs in humans is consistent with the animal evidence [12,16]. Other long-term consequences from early life DEHP exposure may include disruption of female traits as well as neurodevelopment from in utero exposure [53,54], and longlasting effects on immune system [55] and reproductive organ development [56] from postnatal exposure. Metabolic immaturities may also play a role in early life vulnerability to DEHP. Its metabolic disposition involves esterase cleavage to MEHP, an active metabolite, in intestines, liver and other organs. The active metabolite is oxidized to a series of metabolites that are more readily excreted in urine and that are substrates for conjugation with

glucuronide to further facilitate excretion in vitro studies show that the cytochrome P-450s primarily responsible for MEHP oxidation in human tissues are CYPs 2C9 and 2C19 [57]. At birth human liver is immature with respect to the content of these CYPs but develops rapidly in the first months of life; for CYP2C9 adult levels of activity can be reached by 6 months of age while maturation appears to be slower for CYP2C19. During this period CYP2C9 and 2C19 activities are highly variable across individuals [58]. Glucuronidation is also immature at birth and the first several months of life [59]. Therefore, the metabolic clearance processes of CYP-mediated oxidation and Phase II conjugation may be immature in early life and lead to a metabolism-based vulnerability to DEHP. However, this needs further exploration.

#### 4.1.2. Life Stages Targeted in the Current Exposure Assessment

**In Utero Development:** Pregnancy is selected because it contains the most sensitive window of exposure to DEHP. A masculinization programming window has been identified as the first trimester, weeks 9–14 of human gestation, with this potentially the most sensitive period for DEHP's effect on in utero male development [12,60]. This suggests that peak exposures to DEHP of just a week or two duration during this masculinization window may be sufficient to affect development. Thus, one needs to consider whether there can be episodic sources of exposure during pregnancy.

The main exposure pathways during pregnancy for the general public are dietary, house dust and indoor air. Drinking water exposures are also evaluated. DEHP is not known to be commonly used in personal care products.

**Breast-Fed Infant:** DEHP's frequent detection in breast milk makes this a priority life stage of analysis. Breast fed infants ingest mother's milk as the major source of fluid and nutrition for 0–6 months postnatal so that is the exposure period chosen for this age group [18]. As stated above, postnatal DEHP exposures may represent an important window of vulnerability to DEHP endocrine effects, as well as a period of reduced metabolic clearance.

The case study for this life stage considers breast milk ingestion and exposure to indoor air (inhalation, dermal) as the most important pathways to quantitate. We are not including formula-fed infants in this analysis but a similar approach can be used as described herein. The evidence that breast milk and formula have similar DEHP concentrations and the removal of DEHP from baby bottles suggest that exposures to formula-fed and breast-fed infants will be comparable.

**Toddler:** This life stage involves behaviors (crawling, exploratory behavior, mouthing) that lead to greater contact with house dust and contaminants in consumer products; this is also a potentially vulnerable life stage to DEHP's endocrine disruptive effects.

The case study for this life stage considers diet, house dust ingestion, indoor air (inhalation and dermal), mouthing of objects, dermal contact with shoes and clothing and water ingestion to be pathways for more detailed consideration.

**Adolescence:** for brevity this age group is not a focus of the current case study. This is a potentially important window for endocrine disruptors due to important developmental events surrounding puberty. Phthalates are known to be present in personal care products, which can be used at a particularly high rate during this age window. However, the type of phthalates used in cosmetics, fragrance, lotions, nail polish and hair products typically does not involve DEHP. The exposure profile for pregnancy may be a reasonable approximation for this age group as well, although as shown in Figure 1, biomonitoring results amongst 6–11 years old children are consistently higher than in adults.

The behaviors associated with the life stages modeled may have a degree of overlap as exemplified by the fact that children may continue to breast feed into the toddler years and ingestion of non-food items may begin before that time. The calculations presented below illustrate the key exposures for pathways which drive the exposure for a particular life stage without including minor pathways.

#### 4.2. Phase 2. Calculating Exposure Doses via Individual Pathways and Estimation of Aggregate Exposure

The second portion of the children's exposure methodology is the estimation of exposure doses via well-defined pathways with estimation of aggregate dose across all pathways. This case study focuses on central tendencies, average or median estimates of exposure. This provides a useful survey of the key pathways and sources. However, for risk assessment a probabilistic approach such as Monte Carlo analysis may be more informative to show a more complete distribution of exposure relative to a health benchmark. The exposure estimates shown are either extracted from other studies as for example DEHP dietary intakes (Table 1), or calculated by us as indicated in the text.

**Table 1.** Dietary Exposure Estimates for diethylhexylphthalate (DEHP) from Various Studies.

Study	Population	Foods Considered	Estimation Method	Exposure Estimate (ug/kg/d)
Sioen et al. 2012 [11]	Preschool children in Belgium	Market basket survey of DEHP content of 550 food items	DEHP content × ingestion rate of food	P50: 3.7 P99 (worst case <sup>a</sup> ): 37
Beko et al. 2013 [7]	3–6 years old German children	No dietary analysis	total intake from urinary biomarker—indoor pathways	Average: 6.8
Rudel et al. 2011 [44]	5 San Francisco families, 20 individuals, broad age range	Dietary intervention for 3 days, avoiding packaged foods	Drop in urinary biomarker during intervention converted to intake dose	3.0
Sioen et al. 2012 [11]	Adults in Belgium	Market basket survey of DEHP content of 550 food items	DEHP content × ingestion rate of food	P50: 1.5 P99 (worst case <sup>a</sup> ): 19
CPSC 2010 [10]	6 month to 4 years old children	Canadian market basket survey of 98 foods, Chan and Meek 1994	DEHP content × ingestion rate of food	5.0
CPSC 2010 [10]	Adults	7 day sampling of diet from 50 adult Germans	DEHP content of food × ingestion rate of food	3.95
Sakhi et al. 2014 [61]	Adults	Norwegian market basket survey of 10 food categories	DEHP content of food × ingestion rate of food	Average: 0.4 95th %: 0.8

<sup>a</sup> DEHP concentration was maximum detect for each food item.

#### 4.2.1. Breast-Fed Infant

USEPA's Exposure Factors Handbook [18] has a recommended rate of breast milk ingestion that ranges from 510 mL/day (150 mg/kg body wt/day) in the first month of life to 770 mL/day (110 mL/kg/d) in the 3–6 month period with further declines from there. One can consider the ingestion rate for 1–3 month old (690 mL/day or 140 mL/kg/d) as an estimate of the average of this period of peak breast milk ingestion. We have combined this ingestion rate with the median concentration of DEHP + active metabolite MEHP detected in breast milk (6.2 ug/L, [41]) to yield an estimate of breastfeeding exposure of 0.87 ug/kg/d (0.14 L/kg/d × 6.2 ug/L) for 1–3 month old infants. An upper bound estimate based upon the 95th percentile of the DEHP and MEHP detections in breast milk is 23.8 ug/L [41] corresponding to 3.3 ug/kg/d. This is similar to the range of DEHP ingestion calculated by Kim et al. [42] based upon sampling of 62 breast milk samples from Korea, 0.9 to 6.5 ug/kg/d.

Exposure to DEHP in indoor air is a potentially important pathway for this lifestage. Indoor air DEHP concentrations have been measured in studies of homes and day care centers as well as been the subject of indoor fate and transport modeling [40,62–64]. A central tendency estimate of 0.1 ug/m<sup>3</sup> and an upper bound of 1 ug/m<sup>3</sup> can be derived from Xu et al. [27] based upon modeling results for indoor air checked against a range of results from indoor air studies. We combine this estimate with exposure parameters available for the 0–6 month old infant [46] to derive an inhaled dose of 0.033 to 0.33 ug/kg/d. This calculation used a respiration rate of 4 m<sup>3</sup>/d, an absorption rate of 50% via inhalation and a body weight of 6 kg [18]. This DEHP inhalation dose agrees with estimates provided by Xu et al. [27] for inhalation exposure of young children to indoor air DEHP. Dermal exposure to airborne DEHP can also occur by deposition and partitioning of DEHP into skin followed by transdermal uptake. Xu et al. [40] estimated this to be approximately double the daily

dose from inhalation of the same DEHP indoor air concentration. Thus, dermal uptake from vapor phase DEHP can be estimated to be 0.07 to 0.7 ug/kg/d.

Other exposures for this age group are less well defined but may contribute to DEHP exposure, including mouthing of objects. This pathway is discussed in detail in the next section for toddlers. Since data presented in USEPA's Exposure Factors Handbook [18] suggest mouthing of objects in 3–6 month old occurs at a frequency consistent with older children the pathway estimates for toddlers are applied to this lifestage as well. Since this age is prior to the initiation of crawling, house dust ingestion is not considered to be an important pathway but may not be absent.

The estimates of daily exposure over the first 6 months of life for a breastfeeding infant are summarized in Table 2. These results suggest that breastfeeding is not a key driver of exposure as mouthing of objects can be more important at least for the 3–6 month old infant. Overall, breastfeeding infants are expected to have lower DEHP exposure than toddlers.

Formula-fed infants are not included as a separate analysis because of the evidence cited above that formula and breast milk have similar concentrations of DEHP.

**Table 2.** Summary of DEHP Exposure Estimates (ug/kg/d) Across Pathways in Comparison to Biomonitoring-Based Exposure Estimates <sup>a</sup>.

Exposure Pathway	Pregnancy	Breastfeeding	Toddler
Diet	1.5	Not applicable	5.0
Breastfeeding	Not applicable	0.9	Not applicable
Inhalation indoor air	<0.1	0.1	0.1
Dermal indoor air	<0.1	0.25	0.25
House dust ingestion	0.16	Not applicable	2.6
Mouthing objects	Not applicable	3.9	3.9
Drinking water	<0.1	Not applicable	0.13
Pathways Total	1.7	5.15	12.0
Biomarker-based Estimates	50th % = 1.43 <sup>e</sup>		7.4 <sup>b</sup>
of Total DEHP Exposure	Max = 17.5 <sup>e</sup>	Not available	8.1 <sup>c</sup>
			2.8 <sup>d</sup>

<sup>a</sup> All data are central tendency estimates (means, medians) rather than upper percentiles. See text for derivation and sources of estimates for pathways approach; <sup>b</sup> Estimate is for a slightly older age group, 3–6 years old, Beko et al. 2013 [7]; <sup>c</sup> Estimate for 30 children in Taiwan, 2–3 years of age, samples from 2002–2003, Lin et al. 2011 [65]; <sup>d</sup> Estimate for 19 children in USA, age range 12 to 18 months, sampled in 2000, based upon calculations provided in Beko et al. 2013 [7]. <sup>e</sup> Estimate from urinary metabolites in 209 adults of both genders in Belgium, Dewalque et al. 2014 [66].

#### 4.2.2. Toddler: 1–2 Years of Age

This age group primarily receives dietary exposure to DEHP from solid foods and nutritive liquids rather than from breast milk. Inhalation and dermal uptake from indoor air can be assumed to be similar to the earlier age estimate as both respiratory rate and body mass will be proportionately increased. However, two new exposure routes, mouthing of non-food objects and house dust ingestion are added. The dietary, house dust, mouthing and drinking water exposures are summarized below.

##### Dietary

DEHP is a common contaminant of the human diet with analysis of 8 phthalates in over 550 different food items in Belgium finding the concentrations of DEHP were typically more than an order of magnitude higher than the other phthalates [11]. This study provided aggregate estimates from all foods for children ranging from 3.4 (50th percentile) to 37.5 ug/kg/d (worst case, highest concentration for each food item) with bread (34% of total), fruits (12.5%) and processed meats (7.4%) being the leading source categories [11]. These calculations involve intake rates per body weight for a variety of food items in children, information that can be obtained from USEPA's Exposure Factors Handbook [18]. A more detailed analysis of the sources of DEHP in samples of

commercial bread found that the packaging material (e.g., plastic vs. paper bag) had less to do with contaminant levels than did factors specific to a particular bakery location [64]. The authors surmise that DEHP contamination of kitchen bakeware and starting ingredients (e.g., flour) are likely to be most important [64]. As shown in Table 1, the Belgium dietary estimates for children are similar to results obtained in studies from Canada, Germany and the US. The Rudel et al. [44] study of San Francisco families was a dietary intervention study in which fresh foods with minimal packaging were replaced for conventional foods over a 3 day trial. The decrease in urinary DEHP metabolite was substantial suggesting that a major portion of dietary intake can come from coatings and packaging used for food items.

### Drinking Water

Children's ingestion of DEHP in drinking water was estimated based upon a central estimate drinking water concentration of 2 µg/L in public supplies [48] combined with parameters for this age group: 95th percentile water ingestion rate of 0.89 liter per day for a 11.4 kg body weight [18].

### House Dust

While phthalate release from flooring, wall treatments, shoes and other household items may occur during normal wear and tear, the driving force for their release is vaporization to indoor air [40]. DEHP has a low vapor pressure but it is loosely bound to plastic polymers and given enough surface area (e.g., as in vinyl flooring), there can be a steady release to indoor air. However, the low vapor pressure causes DEHP to adsorb onto particulates and thus partition into house dust. This reservoir can accumulate to relatively high concentrations. A survey of French residential indoor environments found DEHP to be the highest ranked pollutant based upon concentrations found and potential health effects. The mean house dust concentration was 505 µg/g [8] while a similar mean value was found in a survey of German homes [67]. A compilation of recent US studies found a mean of median DEHP concentrations across studies of 383 µg/g with a maximum detected of 6783 µg/g [68]. A phthalates risk assessment set a default house dust ingestion rate for young children at 100 µg/d [9], somewhat less than the assumption for outdoor soil given that children and the objects they encounter will tend to be less dirty indoors than out. This ingestion rate is also consistent with upper bound values suggested by USEPA for indoor dust ingestion [18]. We calculated house dust DEHP dose from this exposure rate combined with the central and upper bound (maximum) for recent US studies to yield 2.6 to 45 µg/kg/d DEHP ingestion from house dust. An estimate for this exposure pathway was provided by CPSC [10] as being 6.6 µg/kg/d for children between 7 months and 4 years of age. In contrast, a study of Danish children at home and in day care environments estimated a dust ingestion dose of only 0.51 µg/kg/d [7].

Another approach to monitoring of indoor dust was an evaluation of the estrogenic potency of the dust collected at Belgium kindergardens [69]. DEHP was detected in every sample and its concentration was correlated with the estrogenic potency of the samples.

### Mouthing of Objects

The repetitive placement of nonfood items in the mouth can lead to contaminant exposure both due to the dust/dirt on the object and because of the potential for saliva to dissolve chemicals from the product. DEHP and other phthalates are not tightly bound into the polymer or resin in which they reside and so can be subject to extraction in a child's mouth. Even though DEHP has been phased out of children's toys and products, it is still present in a variety of consumer items that children may mouth such as packaging, rainwear, fabric and upholstery. In addition, toys that were sold prior to the phase out may still contain DEHP. Based upon a literature review of mouthing behavior and saliva extraction, Heiland et al. [70] estimated young German children to have on average 3.9 µg/kg/d ingestion of DEHP from mouthing household products with this ranging up to a 95th percentile of 10.8 µg/kg/d [70]. This exposure is expected to be greatest in 0.5–2 years old due to their high degree of exploratory behavior at floor level, although the USEPA Exposure Factors

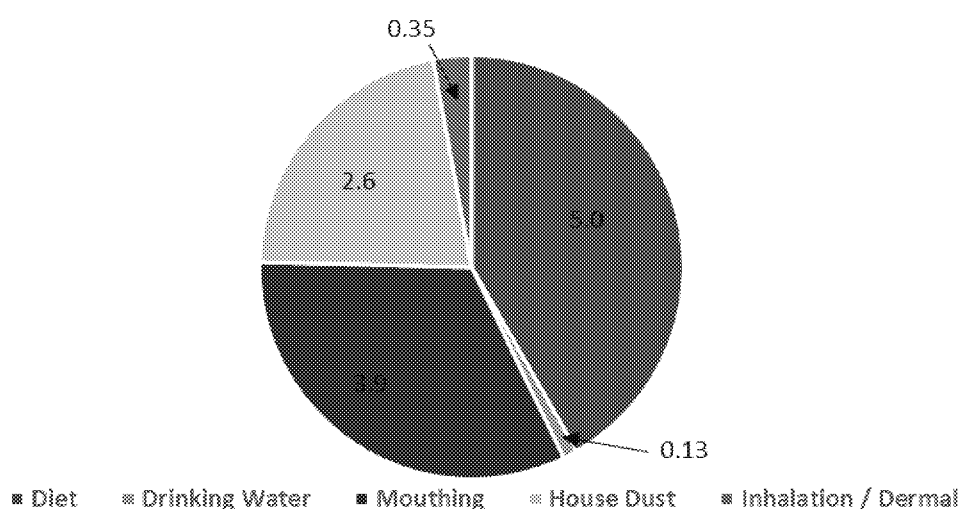
Handbook indicates mouthing behavior frequency in 3–6 month old that is similar to older children [18]. A report by the Consumer Product Safety Commission [10] provided estimates for DEHP mouthing exposure from toys ranging from 10 to 75  $\mu\text{g}/\text{kg}/\text{d}$ ; however, these estimates were from pre-2000 studies and so would not reflect the removal of DEHP from children's products that has since occurred. These estimates for DEHP are compatible with estimates for DINP, a phthalate plasticizer that has replaced DEHP in many items and for which extensive exposure assessment involving the mouthing pathway has been conducted [71]. The anticipation is that DEHP exposure from mouthing of objects has declined and will continue to do so as a result of the phase-out of DEHP from children's toys and related products.

#### Dermal Exposure from Clothing and Footwear

Phthalates are a major ingredient of PVC-based sandals and other footwear, and can also be present in clothing, particularly rainwear. CPSC [10] provided estimates of total phthalate intake from dermal absorption from footwear and rainwear to be 17 (low estimate) up to 420 (high estimate)  $\mu\text{g}/\text{kg}/\text{d}$  for 1–3 years old children. These estimates were not specific to DEHP but show that high phthalate exposure is possible from these sources and could be a significant contributor to this exposure assessment if they contain DEHP. Analyses of children's footwear from the State of Washington described below found most samples to be low in DEHP ( $N = 12$ ).

#### Summary for Toddlers

Figure 2 and Table 2 present the daily dose of DEHP from different sources, showing that diet and mouthing of objects can have the largest contribution followed by house dust ingestion and then the remaining pathways. The aggregate central estimate exposure for these pathways is 12  $\mu\text{g}/\text{kg}/\text{d}$ .



**Figure 2.** DEHP Exposure Pathways in Toddlers Based Upon Central Estimates of Daily Exposure. Unit:  $\mu\text{g}/\text{kg}/\text{d}$ . Numbers are estimates of dose for each pathway.

#### 4.2.3. Pregnant Women

Adult women will receive DEHP exposure via the diet, indoor air and house dust. A variety of phthalates can be present in personal care products (lotions, deodorant, sunscreen, perfume) but for the most part DEHP is not commonly added as an ingredient to these products. Given that a relatively short window of maximal vulnerability to the in utero endocrine disruption effects of DEHP may exist, it is also worth considering whether there could be large seasonal or day-to-day fluctuations in DEHP exposure.

Dietary exposure is considered the main exposure route in adults [72]. Table 1 shows a variety of estimates of adult dietary intake across several European surveys, with a dietary intervention study in the US providing additional information. The results range from <1 to approximately 5 µg/kg/d with the most robust study, involving 550 food items in Belgium estimating a mean of 1.5 µg/kg/d and an upper bound of 19 µg/kg/d [11]. This set of estimates is used for the current exposure assessment, though there is some uncertainty in this because pregnant women may have different consumption rates and patterns than non-pregnant adults [73].

The inadvertent ingestion of house dust by adults is assumed to occur at a rate of 30 mg/day as per the recommendation in the Exposure Factors Handbook [18]. Based upon a central estimate of 383 µg/g DEHP in house dust (see above) and 30 mg dust ingestion per 73 kg body weight, we estimate 0.16 µg/kg/d house dust DEHP ingestion exposure. As described above for toddlers, the inhaled dose from vaporized DEHP and the dermal absorbed dose are expected to be well below the house dust ingestion dose and thus well below 0.1 µg/kg/d.

Drinking water intake of DEHP is expected to be very low (<0.1 µg/kg/d) based upon a water concentration of 2 µg/L as described above with the 95th percentile ingestion rate of 2.59 liters for a 73 kg body weight for mid-pregnancy [18].

The overall DEHP intake estimate for pregnant women (and adults in general) is thus expected to be 1.7 µg/kg/d, composed primarily of dietary intake. In terms of potential short-term peak exposures that may be relevant to a high vulnerability period of pregnancy, time series data within single individuals indicate an ICC of 0.1 to 0.3 for DEHP suggesting large temporal fluctuations [49]. Since adult exposure is driven by dietary intake, one might consider the upper bound estimate of 19 µg/kg/d identified by Sioen et al. [11] as the maximum exposure possible during a critical window of in utero development for consideration of short-term risk to development. However, this is highly uncertain as more detailed dietary intake data are needed in pregnancy to understand the day to day and week to week variability possible in DEHP intake.

#### 4.3. Phase 3. Cross-Check Against Urinary Biomonitoring Estimate of Total Daily Dose

DEHP metabolites have been quantitated across adult populations in Europe and the US with methodologies originally developed by Koch et al. [74] used to convert urinary metabolite data to intake dose. The biomonitoring based quantitation of total exposure provides a useful comparison because it is completely independent of the pathways analysis, relying upon urinary concentrations rather than environmental media concentrations and intake rates. The biomonitoring approach has several uncertainties, such as potential age-related variability in the metabolism and excretion profile of DEHP, whether to use creatinine or urine volume to estimate total DEHP metabolite excretion, and reliability of a single urine sample to be predictive of long-term exposure. However, comparison of aggregate dose estimates via the pathways-based approach and the biomonitoring based approach provides a useful cross-check on the robustness and accuracy of the exposure assessment. If the biomonitoring based approach yields considerably higher dose estimates, then there may be pathways that have been missed or underappreciated. If the pathways-based approach is substantially higher this may reflect different time periods from which the pathways vs. biomonitoring studies were conducted. This is an important consideration for DEHP as exposures have been falling over the past decade (Figure 1); it is also worth considering whether the pathways and biomonitoring based results represent the same percentile of their respective distributions (e.g., comparing medians to medians).

##### 4.3.1. Breastfed Infants

DEHP biomonitoring data for this lifestage were not found and so the pathways-based estimate is the only aggregate dose currently available; thus a Phase 3 comparison between pathways estimate and biomonitoring-based estimate is not possible for this lifestage.

##### 4.3.2. Toddlers

Table 2 provides several biomonitoring-based estimates of total exposure relevant to this age range. The most robust dataset [7] is for somewhat older children but is a useful starting point for this comparison. Beko et al. [7] sampled the urine of 441 3–6 years old Danish children in 2008–2009. This study found that the mean biomonitoring-based dose was 7.4  $\mu\text{g/kg/d}$  (median 4.42, 95th % 16.9). A smaller study of Taiwanese children, aged 2–3 found a similar central tendency 8.1  $\mu\text{g/kg/d}$  [65]. An apparent outlier value is from a small study of children in the US sampled in 2000 which found only 2.8  $\mu\text{g/kg/d}$  (estimate provided in Reference 7). The Beko et al. dataset [7] may provide the best comparison because of its large number of participants and relatively recent sampling period, although the age group is slightly older than what was used for pathways calculations. Comparison of total DEHP exposure by the pathways based (12  $\mu\text{g/kg/d}$ ) and biomonitoring-based (7.4  $\mu\text{g/kg/d}$ ) approaches yields a close match suggesting a reasonable degree of confidence in the central tendency estimate of DEHP exposure for this age group, especially considering that the slightly younger ages used in the pathways analysis are likely to spend more time mouthing objects as compared to older children [18].

#### 4.3.3. Pregnant Women

Table 2 provides a biomonitoring dataset for adults which converted urinary DEHP metabolites to intake estimates across 209 adults in Belgium [66]. This resulted in an estimate of 1.43  $\mu\text{g/kg/d}$  (50th percentile), ranging up to a maximum of 17.5  $\mu\text{g/kg/d}$ . These estimates closely match the pathways estimates of DEHP exposure in adults (Table 2) suggesting a reasonable degree of confidence in the aggregate and pathways estimates of adult DEHP exposure.

#### 4.4. Phase 4. Identification of Key Pathways and Sources of Exposure

DEHP exposure in children and adults has been declining over the past decade but is still considerable and potentially higher than many other common household contaminants. If opportunities are sought for further reductions, a first step is to use the exposure assessment to look upstream at potential sources. The pathway analysis for toddlers, the lifestage with the highest exposure estimate in the current analysis, is summarized in Figure 2. This assessment suggests three areas of primary importance with respect to DEHP exposure in toddlers: diet, mouthing behavior and house dust. These represent three separate source areas for DEHP entry into the environment of a toddler. Similar analyses can be conducted for pregnant women (diet as leading exposure pathway) and breastfeeding infants (mouthing behavior as leading exposure pathway).

##### 4.4.1. Potential Dietary Sources

Food is the predominant source of DEHP exposure in adults and toddlers. DEHP is fat soluble with evidence of its achieving relatively high concentration (several ppm) in high cream content and other fatty foods [75]. Investigation of dairy production found that DEHP most likely enters in several ways including via trace contaminants in grain fed dairy cows, via the machines used for milking, and at the retail level, via the containers used to ship and package milk and other dairy products [76]. In dietary studies, DEHP exposure tended to be greatest from bread, fruit products and processed meats with many foods making small contributions [11]. Similarly, DEHP intake in Norwegian adults was estimated to come from grains and meat products as the two highest source categories [64]. Examination of the sources of DEHP in bread found that it was not related to the packaging used for the bread but was bakery specific, possibly as a result of contamination of flour and/or baking equipment [64]. The dietary intervention study in US families found that packaged foods can be an important source of DEHP in contrast to fresh and unprocessed foods which involved less DEHP and BPA exposure [44].

Overall, these studies do not indicate a particular consumer strategy for reducing DEHP dietary intake although in theory somewhat less exposure may occur by eating lower fat foods and foods involving less processing and packaging. However, DEHP contamination of foods is widespread

and at a generally low level so that it would be difficult to single out specific foods or categories of food for reduction.

#### 4.4.2. Potential Contributors to House Dust

DEHP in house dust comes from the many sources of DEHP around the home. Modeling studies have shown that the surface area of vinyl flooring is a key factor in the volatilization release of DEHP into house dust [40]. Other DEHP sources around the home which can contribute high surface area for volatilization are shower curtains, synthetic Christmas trees, vinyl wall coverings, vinyl table cloths and plastic carpet backing. More data are needed regarding the frequency and concentration range for DEHP in these products and whether over time the trend is for the use of replacement phthalates. The ongoing presence of DEHP in house dust could represent old sources from materials that had been purchased years ago and are still in the home. Alternatively, contributions to house dust DEHP may stem from new materials that were recently purchased. Given that young children are especially exposed to chemicals in house dust, the various sources of DEHP merits further investigation. Irregardless of the sources of DEHP in house dust, a simple preventative measure is the frequent cleaning of floors, toys and children's high contact surfaces to minimize children's intake of house dust in general. The less house dust on floors and surfaces, the less it will act as a sink for volatilized DEHP [40] and the less that will be available for children's ingestion. Objects that are mouthed, as discussed in the next section, are also potential contributors of DEHP to house dust.

#### 4.4.3. Potential Sources of Mouthing Exposure

The greatest potential for DEHP mouthing exposure in toddlers is from products designed for children which contain DEHP. However, recent limits on DEHP and several other high concern phthalates from children's products across the United States and European Union have likely decreased the exposure potential from this pathway. For example a 2014 report prepared by the State of Washington [77] tested a limited number of children's products for DEHP content. While we do not have results from earlier years, the Washington report stated that the frequency of detection and levels of detection for DEHP in most children's products were not high, especially in comparison to the DEHP replacement chemical, DINP. For the category "baby accessories" (e.g., teethingers, pacifiers, bottles, bibs) only 2 of 38 samples had detectable DEHP, with these concentrations both below 50 µg/g. Out of 18 "bath accessories" DEHP was detected in 5 samples, with one in particular, a bath book, having a relatively high content (1630 µg/g) but other bath books were non-detect and one rubber ducky was found to contain 58 µg/g. Children's cosmetics had detections in 8 of 26 products with most at relatively low concentration; however, one of three lip gloss samples contained DEHP at 1030 µg/g. Children's footwear contained DEHP in 5 of 12 samples with only one sample above 100 µg/g (ballet slippers at 336 µg/g). These results suggest that the routine use and mouthing of products intended for children can still lead to DEHP exposure but it will be sporadic, highly variable and typically not at high concentration. In contrast to children's products, the packaging of items intended for children can have high concentrations of DEHP. The Washington report shows that the plastic packaging and shrink wrap that children's cosmetics, art supplies, jewelry and toys can contain over 100,000 µg/g DEHP. While such packaging is meant to be discarded after the product is opened, it is possible that the packaging can be a source of DEHP exposure due to mouthing of the item while packaged or because the DEHP leaves the packaging and enters house dust. A 2012 study by an advocacy group in New York State found high levels of DEHP in children's movie themed backpacks and lunch boxes, as well as in rain boots [78]. Thus, while DEHP sources in children's products have decreased there may be opportunities to further reduce DEHP contact and mouthing exposures from products and from the packaging of products that are intended for use by children. Other household products may also be a source of children's mouthing exposure although data on the levels of DEHP in mouthable household items (e.g., clothing, fabric, plastic pens, food packaging containers) are not available.

#### 4.5. Risk Assessment Calculations

The exposure estimates provided in this manuscript are to assist with source apportionment, the goal of the current analysis. Such estimates need to be in common units across pathways so that comparisons can be made and the most important exposures identified. These exposures have been described in this analysis as the average dose on a given day of exposure without considering averaging over longer exposure periods, a distribution of exposure percentiles, or different time units as may be needed for risk assessment. Dose calculations in risk assessment are endpoint specific (cancer different than non-cancer). Cancer potency values are based upon a lifetime average daily dose: the animals in the cancer bioassay may have gotten less cancer if they were not dosed for their entire lifespan. The fact that the dosing in animal cancer bioassays doesn't actually begin until the animals are weaned has led to the development of age dependent adjustment factors (ADAFs) that can account for this extra exposure period and early life vulnerability [20]. Thus, the calculation of exposure dose in children (e.g., breastfeeding infant, toddler) has to be considered within the context of what is the dose over the critical window of heightened cancer risk vulnerability (e.g., the first 2 years of life) so that the estimation of early life cancer risk (exposure dose \* adult-based cancer slope factor \* ADAF) can be separately calculated and added to the cancer risk from other life stages. For non-cancer endpoints, the assumption is that a chronic period of exposure is necessary to compare the exposure rate to the dose associated with chronic non-cancer risk, the reference dose (RfD). Since less than chronic periods of heightened exposure in early life (e.g., breastfeeding infant, toddler) do not match with the chronic period associated with the RfD, a time weight averaging calculation is often used to relate the exposure scenario back to the RfD. Further details on exposure calculations for risk assessment can be found in documentation provided by USEPA [19].

### 5. Summary and Conclusions

The case study presented for the phthalate plasticizer DEHP provides a 4 step methodology for understanding children's sources of exposure to a ubiquitous environmental contaminant. DEHP is a good case study because it has sufficient environmental and biomonitoring data to enable comparison of the pathways-based and biomonitoring-based approaches to aggregate exposure assessment in pregnant women and children. A 5th step (calculations for risk assessment) is also described if one is conducting the exposure assessment as part of a DEHP risk assessment. The methodology and its application to DEHP are summarized as follows:

*Prioritize exposure pathways and age groups for quantitative analysis:* the DEHP analysis identified breastfeeding infants, 1–2 years old toddlers, and pregnant women as key life stages for exposure assessment. Pathways specific to each lifestage were identified for quantitative analysis. Other early life periods may also represent important windows of vulnerability or may be important to add into a cumulative exposure and risk assessment. However, for the purposes of the current case study, the selected age groups illustrate the range of exposure considerations and doses of DEHP for early life periods.

*Estimate pathway-specific and aggregate exposure:* Table 2 provides exposure estimates for a variety of exposure pathways for the life stages identified in Step 1. The aggregate exposure estimate is greatest for toddlers followed by breastfeeding infants and then pregnant women. The pathways of greatest potential exposure for toddlers are shown in Figure 2 and Table 2 as being diet, mouthing of objects and ingestion of house dust. The other pathways considered, inhalation of vapor phase DEHP, dermal uptake, and drinking water ingestion, were found to make minor contributions. The pathway breakdown for the other lifestages is shown in Table 2 with diet being the dominant pathway for pregnant women and mouthing of objects the dominant pathway for breastfeeding infants.

*Compare the estimate of aggregate exposure from pathways analysis to dose estimates from biomonitoring studies:* the urinary biomarkers for DEHP exposure have been assessed in a variety of studies with several of these studies converting the biomarker result to total aggregate DEHP intake. These biomonitoring-based estimates of total exposure have been compared to the pathways-based estimates in Table 2 for toddlers and pregnant women. In spite of very different methodologies and

a variety of uncertainties in the two approaches, their estimate of total aggregate DEHP exposure was very similar. This provides a reasonable degree of confidence in the exposure estimates for these life stages. These aggregate doses are central tendency estimates; additional analysis would be needed to explore whether the pathways and biomonitoring-based approaches are in agreement for upper bound estimates of exposure, although there is no reason to suspect that they would not. Comparison across different quantitative approaches was not possible for breastfeeding infants due to lack of data.

Determine which are the quantitatively most significant exposure pathways and explore which sources of chemical are important to these pathways: the source apportionment focused upon diet, mouthing of objects and house dust as the most important exposure pathways to early life stages. Source apportionment for these pathways is summarized as follows:

I. Dietary sources are widely varied and require further market basket sampling to evaluate trends and food category contributions to total intake. The suggestion from available data is that DEHP exposure is broadly distributed in the diet with a combination of factors (lipid content of food, processing and packaging of the food) having some influence on DEHP content. No clear consumer strategy for reducing DEHP exposure is evident although further analysis of fresh prepared vs. processed/packaged foods may provide a better indication of the importance of these factors [44].

II. Mouthing of objects, both those intended for children's use as well as general household objects, can be ongoing sources of early life exposure. In particular, product packaging can have high concentrations of DEHP and can thus be a source of mouthing exposure. High DEHP content is also possible in children's backpacks and lunch containers. These sources may represent opportunities for reformulating consumer items to decrease children's contact with DEHP.

III. House dust sources are highly varied and appear to include the products and packaging described above as well as general household items which contain DEHP such as vinyl flooring, wall treatments and consumer items such as shower curtains, tablecloths and plastic Christmas trees. Additional data are needed in these source categories to better determine the most quantitatively important sources. Models developed for DEHP release from flooring need to be adapted to other products to understand the relative importance of different source categories. A simple measure to minimize house dust ingestion exposure is public education surrounding the importance of frequent cleaning of floors and high contact surfaces in homes and day care centers.

The result of this methodology for DEHP is the identification of key pathways of exposure along with opportunities for lowering exposure in early life. It has also identified important data gaps and research needs to improve our understanding of the sources of DEHP exposure to pregnant women and children. When needed this approach can be refined using additional life stages [50] and different percentiles of the exposure distribution to explore the variability in exposure and its implications for risk assessment.

This manuscript focused its case study on a well-studied chemical that has a substantial environmental sampling and biomonitoring database. For less well studied chemicals the approach described above can be used to highlight the data that are available and provide exposure estimates and source apportionment to the extent possible. The approach would also be used to identify critical data gaps that prevent a more comprehensive or higher confidence assessment. It may be possible to make a preliminary determination as to whether children's exposures are likely to be disproportionate due to the chemical's presence in breast milk, house dust, foods eaten at higher rate by children, or in consumer products designed for children. Such evidence would further emphasize the need for research into children's exposure pathways and aggregate exposure to that chemical.

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